# Beta-Glucan induced immune modulation of wound healing in common carp (Cyprinus carpio)

**Jiménez, Natalia Ivonne Vera; Nielsen, Michael Engelbrecht; Lindenstrøm, Thomas**

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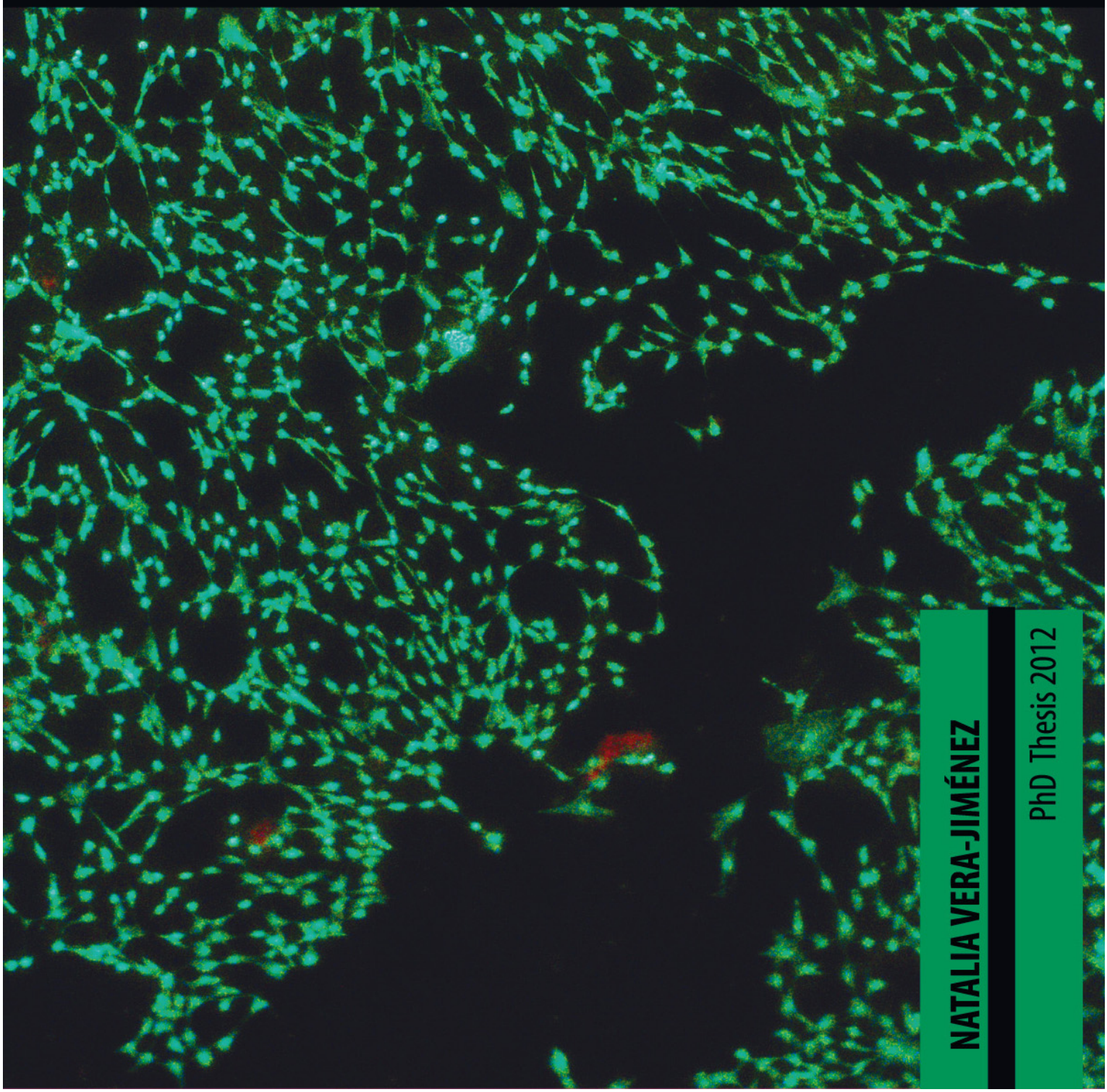
*(Cyprinus carpio)*



NATALIA VERA-JIMÉNEZ

PhD Thesis 2012

# β-glucan-induced immune modulation of wound healing in common carp (*Cyprinus carpio*)

**Natalia Ivonne Vera Jiménez**

**Ph.D. Thesis 2012**

**Supervisors**


| | |
|---|---|
| **Main supervisor:** | **Associate Professor Michael Engelbrecht Nielsen** |
| | Biological Quality group |
| | National Food Institute |
| | Technical University of Denmark |
| **Co-supervisor:** | **Research Scientist Thomas Lindenstrøm** |
| | Department of Vaccine Research and Development |
| | Statens Serum Institute |


**Assesment Committee**


**Senior Research Scientist Christine Lydia Nellemann**

Division of Toxicology and Risk Assessment

National Food Institute

Technical University of Denmark

**Assistant Professor Maria Forlenza**

Cell Biology and Immunology Group

Wageningen Institute of Animal Sciences

Wageningen University

**Associate Professor Søren Skov**

Laboratory of Immunology

Faculty of Life Sciences

University of Copenhagen



"β-glucan-induced immune modulation of wound healing in common carp" Ph.D. Thesis




Cover: Scratch-wounded CCB fibroblasts one day post-wounding. Staining: fluorescein diacetate and propidium ioide.

Back-cover: Fibrosis-like formation of scratch-wounded CCB fibroblasts stimulated with MacroGard® one day post wounding. Staining: fluorescein diacetate and propidium ioide.

# β-glucan-induced immune modulation of wound healing in common carp (*Cyprinus carpio*)

**Natalia Ivonne Vera Jiménez**

Biological Quality group

Division of Industrial Food Research

National Food Institute

Technical University of Denmark

**Ph.D. Thesis 2012**

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## Accompanying Papers

### PAPER I

Vera-Jiménez N.I., Pietretti D., Wiegertjes G.F. & Nielsen M.E. Comparative study of ß-glucan induced respiratory burst measured by Nitroblue tetrazolium assay and Real-time luminol-enhanced chemiluminescence assay in common carp (*Cyprinus carpio* L.). Re-submitted to Fish and Shellfish Immunology.

### PAPER II

Przybylska D.A., Schmidt J.G., Vera-Jimenez N.I., Steinhagen D. & Nielsen M.E. β-glucan enriched bath directly stimulates the wound healing process in common carp (*Cyprinus carpio* L.). Re-submitted to Fish and Shellfish Immunology.

### PAPER III

Vera-Jiménez N.I. & Nielsen M.E. Carp head kidney leukocytes display different patterns of oxygen radical production after stimulation with PAMPs and DAMPs. Submitted to Molecular immunology.

## Summary

Immune modulators are compounds capable to interact with the immune system and to modify the host response. This interaction enhances non-specific defense mechanisms, improving health and promoting survival. β-glucans are glucose polysaccharides present in sea weed, bacteria, fungi and cereal but not in animals. β-glucans are commonly used as immune modulators, but the mechanisms through which the modulation is achieved remains to be understood. Wound healing and tissue regeneration are essential mechanisms to ensure the survival and health of any organism. Studies based in mammalian systems have shown the importance of fibroblasts, macrophages, reactive oxygen species (especially hydrogen peroxide) and certain cytokines during wound healing processes. In fish however, only a few studies have been devoted tissue regeneration and modulation of cell proliferation during wound healing, even though mechanical injury as well as numerous diseases can severely damage fish tissues. The work presented in this thesis examines for the first time the immunomodulatory effects of β-glucans during wound healing processes in common carp.

First, in order to choose the most suitable methodology for the measurement of reactive oxygen species (ROS), the nitroblue tetrazolium assay (NBT) and the real time luminol-enhanced chemiluminescence assay (RT-luminol assay) were compared. Both methodologies successfully detected changes in the production of reactive oxygen species. However, only the RT-luminol assay was able to measure hydrogen peroxide, and allowed the monitoring of the ROS kinetics.

Second, an *in vivo* study was performed to evaluate the immunomodulatory effect of β-glucan during wound healing in carp. Mechanically wounded carps were bath treated with β-glucans or left untreated. The wound healing process was monitored using image analysis and showed that β-glucan bath treatment promoted wound closure in carp. Expression of IL1β and IL6 were increased at day 3 in the site of injure of β-glucan bath treated carps. Furthermore, an increased expression of IL-8 was measured at day 3 and 14 in untreated mechanically wounded carps. Therefore, the β-glucan immune-stimulatory effect on wound healing might be due to the enhancement of an early inflammatory response, with a prompt withdraw of an elevated influx of neutrophils from the wound site. *In vitro* studies showed that direct stimulation with β-glucan did not have any effect in cell proliferation and wound recovery of scratch-wounded *Cyprinus carpio* brain (CCB) fibroblasts cultures. This observation suggests that interaction of tissue-resident leukocytes or other components of the fish immune system

and fibroblasts is required to obtain the immune modulatory effect of β-glucan in wound healing observed *in vivo*.

Third, *in vitro* stimulation of carp head kidney (HK) derived macrophages showed that different ROS patterns are produced after stimulation with PAMPs (β-glucan) and DAMPs (fish fibroblast lysates). β-glucan stimulation of HK derived macrophages resulted in fast and vigorous production of reactive oxygen species, consistent with a pathogen eradication strategy. This response was highly dominated by production of superoxide anion. In contrast, DAMP stimulation led to a slow, subtle but long-lasting production of oxygen radicals dominated by hydrogen peroxide. To determine the effect of hydrogen peroxide release in fibroblast proliferation during wound healing, scratch-wounded CCB fibroblasts were stimulated with different doses of hydrogen peroxide and the wound closure was followed by image analysis. Fibroblast stimulation with low doses of hydrogen peroxide (5 μM) showed a slight increase in percentage of wound recovery, whereas high doses (300 μM) impaired wound closure and caused cell death. The results elucidated the capacity of hydrogen peroxide to influence the fate of tissue regeneration through the establishment of environments suitable for tissue regeneration or oxidative stress.

To conclude, β-glucan treatment enhanced wound closure in carp, probably due to the enhancement of a localized inflammatory response. The wound healing modulatory effect of β-glucan seems to be orchestrated by the immune system, since no direct effect on fibroblast proliferation was observed. Furthermore, production of ROS such as hydrogen peroxide may influence the fate of tissue regeneration, and differences in ROS patterns could be one of the possible ways in which fish alert the immune system to drive the immune response towards pathogen eradication or tissue repair.

## Sammendrag

Immunomodulatorer er stoffer der er i stand til at interagere med immunsystemet og derigennem forandre værtens immunologiske respons. Denne interaktion stimulerer medfødte forsvarsmekanismer og kan derigennem forbedre sundhed og forøge overlevelse af værten. β-glucaner er sukkerstoffer (polysakkarider) der er til stede i tang, bakterier, svampe og korn men som ikke findes i dyr. β-glucaner er kendte immunomodulatorer, men måden hvorpå de modulereimmunsystemet er endnu ikke fuldt forstået. Sårheling og vævsregenerering er væsentlige mekanismer for at sikre overlevelse og sundhed for enhver organisme. Undersøgelser baseret på pattedyr har vist at fibroblaster, makrofager, reaktive oxygenarter (ROS) (især hydrogenperoxid) og visse cytokiner er i forbindelse med sårhelingsprocessen. For fisk er få studier blevet dedikeret til vævsregeneration og modulering af celledelingen under sårheling, dette på trods af at mekanisk skade som følge af håndtering af fisk og sygdomme kan beskadige fiskevæv. I denne ph.d. afhandling undersøges for første gang de immunomodulatoriske effekter af β-glucaner på sårhelingsprocessen i karpe.

For at undersøge hvilken metode der er den bedst egnede til måling af ROS blev et endepunkts nitroblue tetrazolium assay (NBT) og et real-time kemiluminescens-assay (RT-luminol assay) sammenlignet. Begge metoder blev fundet brugbare til påvisning af ændringer i produktionen af ROS. RT-luminol-assayet var ydermere i stand til at måle hydrogenperoxid, samt at overvåge kinetikken i produktionen af ROS.

For at undersøge den immunomodulatoriske effekt af β-glucan under sårheling i karper blev der udført et *in vivo* forsøg. Karper pådraget en mekanisk skade på den ene side blev "badbehandlet" med to forskellige typer af β-glucaner. Sårhelingsprocessen blev fulgt ved hjælp af billedanalyse og det blev fundet at β-glucan badhandlingen signifikant fremmede sårhelingen hos karper. Ekspression af cytokinerne IL1β og IL6 var forhøjet på dag 3 i de sårede β-glucan badbehandlede karper. Endvidere blev der målt en forøget ekspression af IL-8 på dag 3 og 14 i de ubehandlede mekanisk sårede karper. Dette tyder på at den β-glucan immunostimulerende effekt på sårheling kan skyldes en forbedring af det tidlige inflammatorisk respons, med en hurtig reduktion af neutrofile celler omkring sårstedet. *In vitro* undersøgelser viste, at en direkte stimulering med β-glucan ikke havde nogen effekt på celledeling og sårheling i cellekulturer af skadede *Cyprinus carpio* hjerne (CCB) fibroblaster. Denne observation kan tyde på, at der er en vekselvirkningen mellem leukocyter, der er hjemmehørende i og omkring det skadede væv, og det systemiske immunsystemet. Endvidere

synes fibroblaster at være af betydning for den immunomodulerende virkning af β-glucan i sårhelingsprocessen observeret *in vivo*.

Desuden er det blevet vist at *in vitro* stimulering af karpe hovednyre (HK)-makrofager resultere i forskellige ROS reaktioner ved stimulering med PAMPs (β-glucan) og DAMPs (fisk fibroblast-lysater). β-glucan stimulering af HK-makrofager resulterede i en hurtig og kraftig produktion af reaktive iltradikaler. Dette er i overensstemmelse med et forsøg på patogen udryddelse. Superoxid anion var den dominante radikal i forbindelse med dette svar. I modsætning hertil medførte DAMP stimulation til en langsom men langvarig produktion af oxygenradikaler domineret af hydrogen peroxid. For at bestemme betydningen af hydrogen peroxid frigivelse på fibroblast celledelingen under sårheling, blev der i cellekulturer af såret CCB fibroblaster stimuleret med forskellige doser af hydrogen peroxid. Effekten af hydrogen peroxid på celledelingen (sårlukningen) blev overvåget ved hjælp af billedanalyse. Det blev således fundet at fibroblaster stimuleret med lave doser af hydrogen peroxid (5 μM) resulterede i en lille forhøjelse i procentdelen af sårheling, hvorimod høje doser (300 μM) resulterede i nedsat sårlukning og celledød. Resultaterne visualiserede klart evnen af hydrogen peroxid til at påvirke vævsregeneration ved etablering af lokale miljøer enten egnede til vævsregenerering eller oxidativ stress af patogener.

β-glucan behandling intensiverede sårlukning i karper, sandsynligvis på grund af en styrkelse af et lokalt inflammatorisk respons. Den modulerende virkning af β-glucan under helingsprocessen forekommer at være dirigeret af immunsystemet, da der ikke blev observeret en direkte virkning på fibroblast celledeling. Endvidere blev det vist at produktionen af ROS såsom hydrogen peroxid kan påvirke hvorledes vævsregenerering forløber, og således kan forskelle i ROS mønstre være en af de mulige måder, hvorpå fisken alarmerer immunsystemet til at drive immunresponset mod udryddelse af patogener eller vævsreparation.

## Resumen

Los moduladores inmunitarios son compuestos capaces de interactuar con el sistema inmune y modificar la respuesta del organismo. Esta interacción intensifica los mecanismos no específicos del sistema inmune lo cual resulta en una mejora de la salud y un aumento de la tasa de supervivencia. Un tipo de moduladores son los β-glucanos, polisacáridos de monómeros de glucosa que se encuentran en algas, bacterias, hongos y cereales, pero no en animales. Aunque su uso como inmuno-modulador es común, los mecanismos por los cuales se logra dicha modulación todavía no se han establecido en su totalidad. La cicatrización de heridas y la regeneración tisular son mecanismos esenciales para asegurar la supervivencia y la salud de cualquier organismo. Estudios basados en mamíferos han demostrado la importancia de fibroblastos, macrófagos, especies reactivas de oxígeno (especialmente el peróxido de hidrógeno) y ciertas citoquinas durante los procesos de cicatrización. En peces, sin embargo, sólo unos pocos estudios se han dedicado a la regeneración de tejidos y la modulación de la proliferación celular durante la cicatrización, pese a que diversos traumatismos y enfermedades pueden dañar gravemente sus tejidos. En el trabajo presentado en esta tesis, se analiza por primera vez los efectos inmuno-moduladores del β-glucano en los procesos de regeneración tisular en *Cyprinus carpio* (carpa común).

En primer lugar, a fin de escoger la metodología más adecuada que permitiera la medición de especies reactivas de oxígeno (ROS, del inglés *reactive oxygen species*), se hizo una comparación de los métodos de nitroazul de tetrazolio (NBT, del inglés *nitroblue tetrazolium*) y quimioluminiscencia en tiempo real intensificada por luminol (RT-luminol, del inglés *real time luminol-enhanced chemoluminescence*). Ambas metodologías resultaron en la detección de cambios en la producción de ROS. Sin embargo, únicamente mediante el ensayo RT-luminol fue posible medir peróxido de hidrógeno, así como monitorizar la cinética de las reacciones de ROS. En segundo lugar se investigó *in vivo* el efecto inmuno-modulador del β-glucano durante la cicatrización de heridas en *C. carpio*. Estas heridas fueron infligidas utilizando un punzón de biopsia, y se evaluó el efecto de dos diferentes tratamientos de β-glucano en baño. El proceso de cicatrización de la herida se monitorizó usando análisis de imágenes, y mostró que el tratamiento con β-glucano mejora el cierre de la herida. Asimismo, en esta zona se detectó un incremento en la expresión de las citoquinas IL1-β y IL-6 en aquellas carpas tratadas con β-glucano, y una disminución de la quimioquina IL-8 durante el tercer y catorceavo día en aquellas no tratadas. Por consiguiente, el efecto inmuno-estimulante del β-glucano sobre el proceso de cicatrización podría ser debido a un

aumento localizado de la respuesta inflamatoria temprana, y una disminución anticipada de la migración de neutrófilos a la herida. Por otro lado, estudios *in vitro* mostraron que la estimulación directa con β-glucano no tiene ningún efecto en la proliferación celular y la recuperación de la herida en cultivos de CCB (fibroblasto de cerebro de *Cyprinus carpio*) lesionados mediante técnica de rayado (*scratch-wounded*). Esta observación sugiere que la interacción de leucocitos residentes en tejidos u otros componentes del sistema inmune de los peces con fibroblastos es necesaria para obtener el efecto inmuno-modulador del β-glucano en la cicatrización de heridas observado *in vivo*. En tercer lugar, la estimulación *in vitro* de macrófagos derivados de pronefros de *C. carpio* mostró la producción de patrones distintos de ROS tras estimulación con PAMPs (del inglés, *pathogen-associated molecular pattern*) y DAMPs (del inglés, *damage-associated molecular pattern*): a saber, β-glucano y productos de la disrupción de fibroblastos de pescado, respectivamente. La estimulación con β-glucano resultó en una producción rápida y elevada de ROS, consistente con una estrategia de erradicación de patógenos. Tal respuesta fue dominada por la producción de aniones de superóxido. En cambio, la estimulación con DAMPs condujo a una producción lenta y ligera -aunque de larga duración- de ROS, en este caso dominada por peróxido de hidrógeno. A fin de determinar el efecto de la liberación de peróxido de hidrógeno en la proliferación de fibroblastos durante el proceso de cicatrización, se estimuló fibroblastos CCB lesionados mediante técnica de rayado con diferentes dosis de peróxido de hidrógeno, y se monitorizó el cierre de la herida mediante análisis de imagen. La estimulación de fibroblastos con pequeñas dosis de peróxido de hidrógeno (5 μM) resultó en un ligero incremento en el porcentaje de recuperación de la herida, mientras que dosis elevadas (300 μM) empeoraron la recuperación y causaron muerte celular. Los resultados revelaron la capacidad del peróxido de hidrógeno para influenciar el devenir de la regeneración de tejidos a través del establecimiento de ambientes adecuados para la regeneración o el estrés oxidativo.

En conclusión, el tratamiento con β-glucano mejoró el cierre de heridas en *C. carpio*, probablemente debido a una intensificación de la respuesta inflamatoria localizada. El efecto modulador del β-glucano sobre la cicatrización de heridas está directamente relacionado con el sistema inmune, ya que no se observó ningún efecto directo sobre la proliferación de fibroblastos. Asimismo, la producción de ROS tales como el peróxido de hidrógeno puede influir en el desarrollo de la regeneración de tejidos, y la diferenciación en los patrones de ROS podría ser una de las formas con las que el pez induce y dirige la respuesta inmune hacia la erradicación de patógenos o la reparación de tejidos.

# Table of contents

# 1. General introduction:

Wound healing and tissue repair are highly complex and essential processes to ensure the survival and health of any organism (Martin, 1997; Singer and Clark, 1999). The regenerative capacity differs greatly across organs and organisms, and requires the coordinated interaction of different cell types, cytokines, growth factors and inflammatory components (Poss, 2010). Certain lower vertebrates such as teleost fish have greater regenerative capacity than mammals. Therefore, they are used as a model for tissue regeneration in humans. However, the knowledge on cellular and molecular mechanisms involved in these processes is still limited (Poss et al., 2003). Immune-modulators are compounds capable of interacting with the immune system and modify the host response. Hence, they are used to improve health by enhancing non-specific defence mechanisms (Dalmo and Bøgwald, 2008; Meena et al., 2012). In this thesis, the effect of β-glucan, a known immune modulator, is analyzed during the skin wound healing process of common carp. The β-glucan-induced wound closure is evaluated *in vivo*, and some potential mechanisms underlying the immune modulation are studied *in vitro*.

## 1.1 Fish skin structure

The skin constitutes the outer surface of the fish. Its primary function is to serve as a barrier between the organism and the external environment and is metabolically very active (Noga, 2000). Fish skin can roughly be divided in two layers, the epidermis and the dermis, and is fully covered by a mucus layer. In almost all the fish species the epidermis does not have a dead, keratinized surface, in contrast it consists entirely of living cells which are covered by a layer of mucus (Noga, 2000; Whitear et al., 1980). In scaleless regions of carp, the epidermis forms a multi-stratified layer of about 90 µm (Iger and Abraham, 1990). The main structural component of the fish epidermis is malphigian cells. In the superficial layer these cells are usually flattened, while in the basal layer of the epidermis they appear as columnar cells with their long axis perpendicular to the dermis (Aasbakk and Dalmo, 1998; Iger and Abraham, 1990). Malpighian cells are also referred to in the literature as keratocytes (Svitkina et al., 1997), squamos epithelial cells or filament cells (Noga, 2000), and have shown fast motility and even phagocytic activity in Atlantic salmon and common carp. Therefore, they are considered an important cell type during tissue repair processes (Aasbakk and Dalmo, 1998; Iger and Abraham, 1990; Åsbakk, 2001). Goblet cells (also called mucous cells) are scattered

throughout the epidermis, their morphology is rounded and small when they are present in the basal layer, while in the superficial layer the cells are distended and filled with high amounts of stored mucus (Brown and Wellings, 1970; Shephard, 1994). Club cells are large unicellular glands present in the epidermis. In cypriniformes they are filled with a substance that mediates the "fright reaction". In Anguillidae club cells apparently do not mediate a fright reaction, instead they seem to contribute to formed elements in the slime (Henrikson and Gedeon-Matoltsy, 1967b). Granule cells can be found in the epidermis of bony fish, they are classified serous glands (Henrikson and Gedeon-Matoltsy, 1967b). Lymphocytes and resident macrophages can also be found in the epithelia, involved in immune surveillance (Brown and Wellings, 1970).

The dermis mainly consists mainly of collagen. It is separated from the epidermis by the basement membrane, which is composed of fibrillar material and oriented in parallel to the outer surface of the epidermis (Brown and Wellings, 1970). In scaleless regions, the dermis mostly consists of a dense layer of collagen fibres, in which fibroblasts, chromatophores and small blood vessels are embedded (*stratum compactum*). Just beneath the *stratum compactum*, a layer of loose connective tissue (*stratum spongiosum*) with many adipocytes, fibroblasts, collagen fibres and blood vessels is found. Loose connective tissue of the *stratum spongiosum* merges with loose connective tissue associated with the muscle tissue located just below, and no distinct boundary is present (Brown and Wellings, 1970; Ferri, 1982). In regions where scales are present, the dermis shows a superficial region with loose collagen fibres between the overlapping scales (Brown and Wellings, 1970). The elastic system of collagen fibres in the dermis is arranged in a characteristic pattern which provides resistance to mechanical stress (Ferri, 1982).

The mucus layer covering the fish skin is produced by the goblet cells present in the epidermis. Mucus is mainly composed of water, carbohydrates and glycoproteins including mucins (Shephard, 1994). Two different mucins genes, *Muc2* and *Muc5b*, have been found to be expressed in carp. *Muc2* is only expressed in the first and second intestinal segments, and *Muc5b* in skin and gills (van der Marel et al., 2012). The mucus layer prevents the establishment of parasites, bacteria and fungi since it is continuously sloughed off and replaced. Additionally, it contains a variety of biologically active substances such as lysozyme, lectins, proteolytic enzymes, Immunoglobulin M (IgM), and antimicrobial peptides which are constitutively expressed to provide immediate protection to the fish from

potential pathogens (Lemaître et al., 1996; Magnadottir, 2006; Shephard, 1994; Subramanian et al., 2008).

The composition and rate of mucus secretion has been reported to change in response to microbial exposure or to environmental perturbations. Furthermore, the mucus layer has also been related to respiration processes, ionic and osmotic regulation, and locomotion (Bols et al., 2001; Shephard, 1994).

The importance of the skin and mucus layer for the survival of the fish is demonstrated by the fact that substantial losses of mucus or integrity of the epidermis, by disease or injury, results in significant morbidity and mortality (Noga, 2000). The main structure of fish skin is represented in Figure 1.

### 1.2 Fish muscular tissue

In fish, more than 60% of the body weight can be muscle. The fish muscular tissue is formed mainly by two different muscle fibre types: the white and the red fibres (Fernández and Calvo, 2009). These fibres are produced by the fusion of mono-nucleated muscle cells, and are organized in segments separated by connective tissue. The main component of the connective tissue is collagen, helping to maintain the muscular structure and function. White and red muscle fibres differ in their colour, innervations, blood supply, abundance of mitochondria, speed of contraction, fibre size and myosin isoforms (Chong et al., 2009; Fernández and Calvo, 2009). Another cell type present in the muscle tissue is fibroblasts. As main producers of collagen they play an important role for the muscular composition and tissue repair following injury. Furthermore, fibroblasts have been reported to serve as immune sentinels in fish and mammals (Ingerslev et al., 2010b; Smith et al., 1997). Endothelial and blood cells can be found in the muscular tissue and are related to vascularisation. In healthy organisms, immune cells are not normally found in the muscular tissue. However in the presence of infection or tissue damage, different types of immune cells are recruited to the site of injury (Martin, 1997). The main structure of the muscular tissue is represented in figure 1.

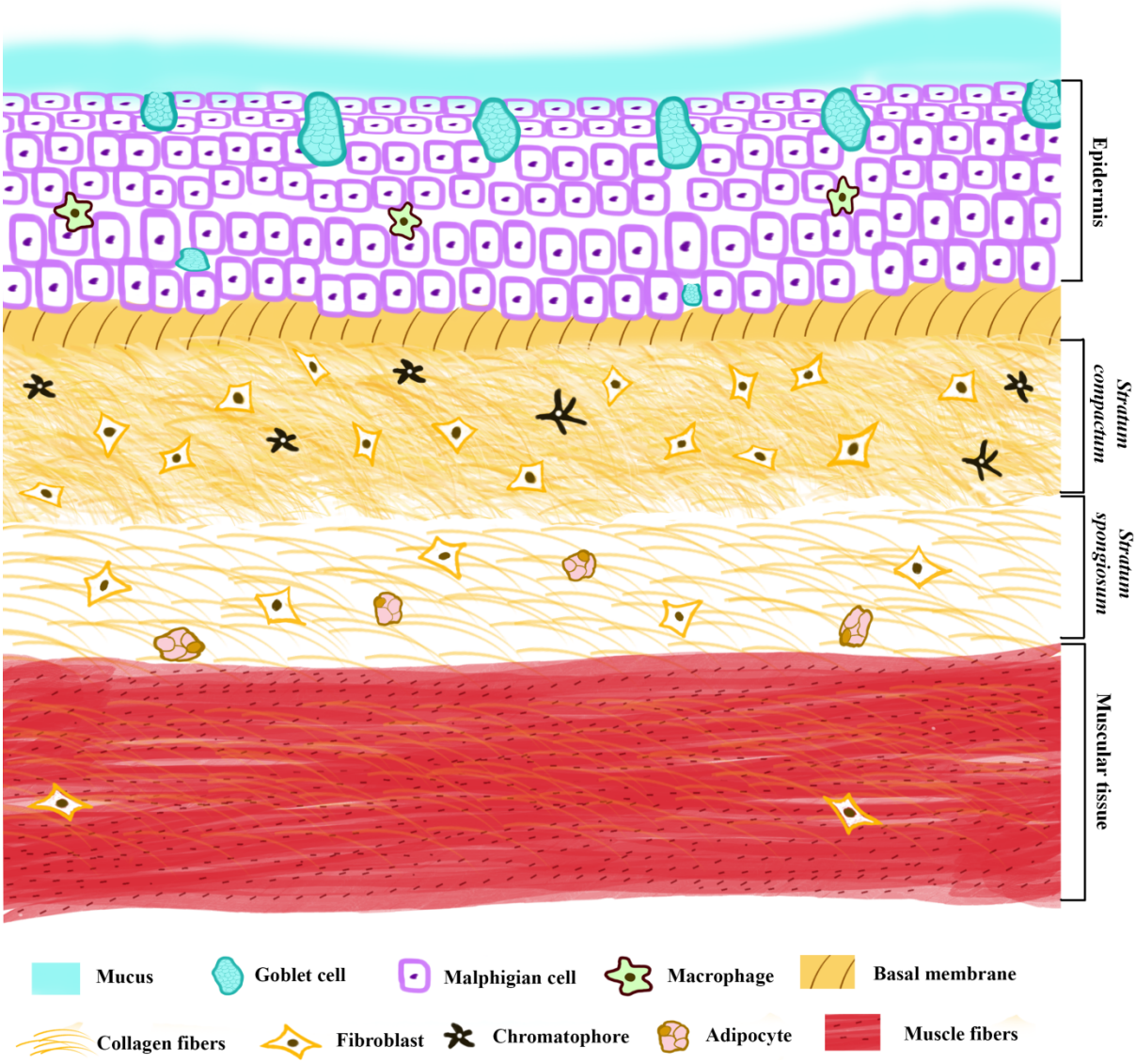


**Figure 1 Schematic representation of the skin and muscular structure in scaless fish.** The skin is the outer surface of the fish and it is covered by a mucus layer. The epidermis is a multi-stratified layer composed mainly by malphigian cells, goblet cells and some macrophages are scattered through the epidermis. The dermis it is separated from the epidermis by the basement membrane, and can be divided in two stratums, the *compactum* and the *spongiosum*. The stratum *compactum* is a dense layer of collagen fibres in which fibroblasts and chromatophores are embedded. The stratum *spongiosum* lies beneath the *compactum* and is composed of loose connective tissue with many adipocytes and fibroblasts. Under the dermis the muscular tissue is found, composed by muscular fibres formed by the fusion of mononucleated muscle cells, some fibroblasts and collagen fibres are also present in the muscle.

### 1.3 Tissue Damage in fish

A wound is defined as a defect in the structural or functional integrity of the tissue, and can be caused by a wide range of insults such as infections and disease, environmental factors or mechanical injury (abrasions and lacerations) (Fantone and Ward, 1982; Frommel et al., 2011; Kovacs and DiPietro, 1994; Noga, 2000). Several infective agents have been reported to affect skin and muscular tissues in carp and other fish species, influencing survival and

quality of the fish meat (Ingerslev et al., 2010a; Jeney and Jeney, 1995; Johnson, 2004). Bacterial pathogens such as V*ibrio anguillarum*, *Aeromonas salmonicida*, *Yersinia ruckeri, Aeromonas hydrophila* (Damsgård et al., 2004; Gudmundsdóttir and Björnsdóttir, 2007; Jeney and Jeney, 1995; Stuart, 1988), fungal diseases such as saprolegniasis, a variety of parasitic infestations (Johnson, 2004) as well as viral diseases such as spring viraemia of carp (SVC) (Ahne et al., 2002) have been related to haemorrhages and necrosis in skin and muscular tissues.

Xenobiotics and other human borne pollutants such as metal ions, pesticides, industrial chemical discharges and even environmental factors like the increase of ocean acidification, have shown to cause mild to severe tissue damage, necrosis and increase of apoptosis in fish and other aquatic organisms (Frommel et al., 2011; Gaber, 2007; Julliard et al., 1996; Lushchak, 2011; Noga, 2000). Furthermore, extensive loss of tissue has been reported in farmed fish as a result of mechanical damage associated with high stocking densities, aggressive interactions, abrasion with nets and cages, fish sorting, and transportation (Ashley, 2007; Ellis et al., 2002).

### 1.4 Cell death

Cell death is an essential and structured process playing a role in morphogenesis, tissue homeostasis, wound healing and elimination of infectious agents (Nelson and White, 2004). Although several types of cell death are known (Fink and Cookson, 2005), two major types have been widely studied: necrosis and apoptosis (Nelson and White, 2004)

Necrosis is usually associated with physical insults, failure of osmotic regulation, ATP depletion, vacuolation of the cytoplasm, breakdown of the plasma membrane and an induction of inflammation around the dying cell attributable to the release of cytoplasmic contents and pro-inflammatory molecules (Degenhardt et al., 2006; Edinger and Thompson, 2004). Apoptosis has been catalogued as programmed cell death and is a well-defined process. It is mediated by a subset of caspases and is characterized by nuclear condensation and fragmentation, cleavage of chromosomal DNA into internucleosomal fragments and packaging of the inert cell into membrane-bound fragments called apoptotic bodies. The apoptotic bodies are recognized and eliminated by phagocytic cells without presence of inflammatory processes in neighbouring cells. An event termed apoptotic necrosis can occur when the apoptotic bodies are not ingested by phagocytes, and their internal contents are released (Degenhardt et al., 2006).

Other ways of cell deaths include: i) Autophagy, featuring the degradation of cellular components within the intact dying cell in autophagic vacuoles, and slight chromatin condensation. Autophagic cells are internalized by phagocytes without the release of pro-inflammatory molecules (Edinger and Thompson, 2004; Fink and Cookson, 2005). ii) Oncosis, is defined as a pre-lethal pathway leading to cell death by cellular and organelle swelling, increased membrane permeability and eventual release of pro-inflammatory cellular contents. iii) Pyroptosis, is defined as a pathway to cell death dependent on the activation of caspase-1, which is not involved in the apoptosis pathway, leading to pro-inflammatory cytokine processing and cellular membrane breakdown (Fink and Cookson, 2005). iv) Necroptosis, is a programmed necrosis by "death receptors" such as tumor necrosis factor receptor 1, and requires the kinase activity of the receptor-interacting protein 1 and 3 (RIP1 and RIP3). Necroptosis participates in pathogenesis of ischemic injury, neurodegeneration and viral infections among others. Its execution involves the active disintegration of mitochondrial, lysosomal and plasma membranes (Vandenabeele et al., 2010). The proteins or products released as a result of any type of cellular death are considered damage-associated molecular patterns (DAMPs), and can be recognized by the immune system (Hansen et al., 2011).

Apoptosis and necrosis have been implicated in tissue damage perpetrated by infectious agents (Falcón et al., 2001; Janda and Abbott, 2010; Law, 2001; Silva et al., 2008), mechanical damage (D'lima et al., 2001; Kurz et al., 2005; Piper et al., 1998; Piper and García-Dorado, 1999; Pollman et al., 1999) and environmental factors (Frommel et al., 2011; Julliard et al., 1996; Noga, 2000).

### 1.5 Tissue repair:

All types of wounds result in the activation of multiple cellular and molecular processes intended to restore the integrity of the skin (Godwin and Brockes, 2006; Mescher and Neff, 2005). As a result, and with direct involvement of the immune system, several overlapping phases including coagulation, inflammation, cell proliferation and tissue remodelling take place (Diegelmann and Evans, 2004; Schaffer and Barbul, 1998).

### 1.5.1 Recognition of damage

Detection of tissue damage in the host is accomplished by the innate immune system, using a series of receptors displayed mainly by the immune cells, which identify the nature of the injury and act towards its repair (Bianchi, 2007; Hansen et al., 2011; Rebl et al., 2010; Zhang and Schluesener, 2006). These receptors are collectively known as the pattern recognition receptors (PRRs) (Hansen et al., 2011). Based on molecular signals, PRRs are able to discriminate between infection, open wounds associated with the intrusion of pathogens in the injury, as well as pathogen-free trauma like mechanical tissue damage or sterile inflammation (Rock et al., 2010; Schreml et al., 2010). Infectious agents display series of specific molecular motifs called pathogen-associated molecular patterns (PAMPs), which are essential for the survival and pathogenicity of the microorganism (Bianchi, 2007). PAMPs are not found in the host, therefore are referred to as non-self molecules (Akira et al., 2006). Some examples of PAMPs include Liposaccharide (LPS), lipoteichoic acids (LTA), lipoarabinomannan and β-glucans, which are signatures of gram-negative, gram-positive, mycobacterial and fungal pathogens, respectively (Dalmo and Bøgwald, 2008; Medzhitov and Janeway Jr., 2000). On the other hand, identification of pathogen-free trauma, such as mechanical damage, is accomplished by the recognition of damage-associated molecular patterns (DAMPs) (Hansen et al., 2011). DAMPs are endogenous molecules which in healthy cells are contained inside the cells and hidden from the immune cells, but subsequently released following tissue injury. DAMPs are also referred to as self-molecules (Chen and Nunez, 2010; Lotze et al., 2007). Some examples of DAMPs from mammalian models are heat shock proteins (HSPs), high-mobility group box-1 (HMBG1), ATP, uric acid, hyaluronic acid and mitochondrial DNA (Chen and Nunez, 2010; Midwood and Piccinini, 2010; Zhang et al., 2010). In fish however, DAMPs have not been studied in depth; but collagen derived proteolytic fragments have been reported to indicate the presence of damage (Castillo-Briceño et al., 2009).

### 1.5.2 Response to tissue injury

Based on mammalian models, once the damage is exerted and recognized, platelets are first recruited to the site of injury due to the coagulation process. Platelets start releasing growth factors such as platelet derived growth factor (PDGF) and transforming growth factor β (TGF-β) which activate local cells like fibroblasts (Diegelmann and Evans, 2004). In a fairly overlapping phase, an inflammatory response is launched with neutrophils being the first

leukocyte lineage arriving to the site of injury, leading to the production of highly microbicidal compounds like reactive oxygen species (ROS) and nitric oxide (NO) (Bianchi, 2007). The main objective of the neutrophil recruitment seems to be the eradication of infectious agents. This recruitment takes place in response to a cocktail of cytokines and chemokines produced by local cells (Gonzalez et al., 2007; Martin and Leibovich, 2005). Next, macrophages get to the site of injury to clear the wound of death cells, and other debris. Macrophages do not constitutively express high levels of cytokines and growth factors in their resting state. However, they are very sensitive to danger signals and become strong producers of these molecules, which can mediate and sustain the inflammation, and activate fibroblasts and endothelial cells (Afonso et al., 1998; Martin and Leibovich, 2005). At a later phase, lymphocytes come to the site of injury, marking the progression of the proliferative and tissue remodelling phase (Diegelmann and Evans, 2004; Wei, 2011). In fish, mobilization of epidermal cells and their phagocytic activity has been reported within the first hours after wounding (Iger and Abraham, 1990). This process overlaps with the inflammatory response, which appears to follow the same phases present in mammals, although thrombocytes instead of platelets (since they are not found in non-mammals) are in charge of the coagulation (Jiang and Doolittle, 2003).

### 1.5.3 Inflammation and fate of tissue remodelling

After wounding, four possible scenarios have been described as outcome: tissue regeneration, tissue repair, fibrosis and chronic wounds (Diegelmann and Evans, 2004; Poss et al., 2003). Tissue regeneration is defined as the replacement of a tissue or structure which has been damaged or lost, with a perfect or nearly perfect result. Tissue regeneration in mammals and higher vertebrates is limited only to a few tissues like blood and liver. In contrast, many lower vertebrates and invertebrates can achieve perfect regeneration of damaged organs or limbs. Tissue repair is the normal result after tissue damage in higher vertebrates, where the wound is closed with reduced scarring (Poss et al., 2003). An imbalance during any of the wound healing phases can lead to fibrosis, which is defined as the replacement of a damaged tissue by a deformed, non-functional and excessively accumulated scar tissue. Finally, an ineffective wound healing response will lead to a chronic wound, which is characterized by an over-abundant neutrophil infiltration, chronic inflammation and constant production of connective tissue degrading enzymes, such as collagenase and matrix metalloproteinases (Diegelmann and Evans, 2004).

The duration and magnitude of the inflammatory response are both key factors determining the outcome of the tissue repair process (Kovacs and DiPietro, 1994). Molecules like ROS and NO produced by neutrophils and other leukocytes during inflammation to eradicate infectious agents often perpetrate damage to neighbouring cells (Martin and Leibovich, 2005). However, their requirement for a normal tissue repair (Kanta, 2011; Niethammer et al., 2009) and their function as fibroblast regulators have been documented (Murrell et al., 1990; Schaffer et al., 1997). Different cytokines produced by leukocytes during the inflammatory response can also influence tissue repair (Werner and Grose, 2003). Mammalian models have thus indicated that the so-called Th1 cytokine profile [interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin-2 (IL-2), IL-12 and IL-18,] generally promotes tissue regeneration or repair with normal architecture. Whereas Th2 cytokine profile (IL-4, IL-5, IL,10 and IL-13) favours fibroblast activation towards higher collagen production rates and fibrogenesis (Mescher and Neff, 2005). In fish, the remodelling phase of tissue repair at molecular level has not been well characterized, although studies in macrophages show that classically activated macrophages, which denotes the activation of the macrophage with microbial motives and Th1 cytokines (M1-type cytokines) such as IFNγ and TNF-α, leads to a strong bactericidal response with high production of ROS and NO. In contrast alternative activated macrophages, implicating activation with IL4 and IL13 from the Th2 cytokine response (M2-type cytokines), leads to the formation of metabolites linked to the production of extracellular matrix, cell proliferation and tissue repair (Forlenza et al., 2011).

### 1.6 Immune modulation:

Immune-modulation has risen as an option to improve fish health and welfare. Thus, fish immune-modulators are being explored and many agents are currently used by the aquaculture industry (Moradali et al., 2007; Sakai, 1999). Overcrowding can negatively affect the health of cultured fish, increasing susceptibility to infections that can lead to tissue damage. Different chemotherapeutic agents and drugs have been used to treat bacterial infections in cultured fish, but the incidence of drug-resistant bacteria and environmental issues have become major subjects of discussion (Douet et al., 2009; Sakai, 1999). Vaccination is also used as a method for prevention of infectious diseases. However, vaccines against several pathogenic agents have not been developed, and the immediate control of all fish diseases and mechanical tissue damage associated with overcrowding is impossible using only vaccines (Ellis, 1988; Sakai, 1999).

Immune-modulators or biological response modifiers are compounds capable of interacting with the immune system and modify the host response. This interaction can potentially improve fish health by enhancing non-specific defence mechanisms, such as phagocytic activity, cytokine production and complement activation (Moradali et al., 2007; Sakai, 1999). The outcome of immune-modulation can vary depending of multiple aspects like dose, route and timing of administration, mechanism of action, site of activity and the compound itself (Moradali et al., 2007).

### 1.6.1 β-glucans

β-glucans are glucose polysaccharides connected by β-glycosidic bonds or D-glucose molecules, which are repetitively linked at specific positions, and often have glucose side chains attached (Chen and Seviour, 2007; Falco et al., 2012) β-glucans are naturally present in different organisms such as sea weed, some bacteria and cereals. However, the most common source of extraction is fungal cell walls (Novak and Vetvicka, 2008). Since β-glucans are non-self molecules, they are recognized as PAMPs, and have shown to have stimulatory effects in the immune system of healthy and immune-compromised organisms (Chen and Seviour, 2007; Kumari and Sahoo, 2006b; Medzhitov and Janeway Jr., 2000; Sahoo and Mukherjee, 2002). Some of the immunomodulatory effects of β-glucan in healthy, immunocompromised, injured or infected fish are summarized in table 1.

**Table 1. β-glucan immunomodulatory effects in fish.**

| Specie | β-glucan | Via | Dose | Challenged | Effects | Reference |
|---|---|---|---|---|---|---|
| Common carp | Baker's yeast-derived | Ip injection | 500 or 1000 µg/fish | +*Aeromonas hydrophila* | ↑ Survival rate<br>↑ Blood leukocyte<br>↑ $O_2^-$<br>↑ Bactericidal activity | (Selvaraj et al., 2005) |
| | | 90 minutes bath | 150, 750 or 1000 µg/ml | +*Aeromonas hydrophila* | No effect reported | |
| | | Oral | 1, 2, 4 % | +*Aeromonas hydrophila* | No effect reported | |
| Common carp | MacroGard ® | Feed supplement | 6mg/Kg/day | + *Aeromonas salmonicida* | ↓ *TNFα1, TNFα2*<br>↓ *IL-1β, IL-6* | (Falco et al., 2012) |
| Common Carp | Baker's yeast extract (7% β-glucan, 10% nucleotides) | Oral | 50mg/kg/day | Only β-glucan | ↑ *IL-1β, TNFα, IL-12p35, IL12-p40,* CXC chemokines, 1day post treatment<br>↓ *IL-1β, TNFα, IL-12p35, IL12-p40,* at 3, 5,7 and 10 days post treatment (pt) | (Biswas et al., 2012) |
| | | | | +*Aeromonas hydrophila* | ↑ Phagocytic activity 1 and 3 days pt<br>↓ Viable pathogen counts | |
| Common carp | MacroGard® | Bath | 0.1 µg/l | Mechanical injure | ↑ Wound closure<br>↑ *IL-1β, IL-6* (1 and 3 d post wound)<br>↓ *IL-8* (3 and 14 d post wound) | Paper II |

| Specie | β-glucan | Via | Dose | Challenged | Effects | Reference |
|---|---|---|---|---|---|---|
| Common carp | MacroGard® | HK leukocytes stimulation (*in vitro*) | 100 µg/ml | Only β-glucan | ↑ ROS<br>↑ NO<br>↑ *IL-1β, iNOS, TNF-α1, TNF-α2, IL-11* | Paper I , Paper III & supplementary data |
| Grass carp | Mycelia of *Poria cocos*-derived | IP injection | 10mg/kg | + Grass carp haemorrhage virus | ↑ Superoxide dismutase and Catalase activity<br>↓ Viral infection | (Kim et al., 2009) |
| Koi carp | Baker's yeast-derived | Oral | 0.5% | Only β-glucan | ↑ Blood leukocyte counts<br>↑Respiratory burst<br>↑Phagocytic capacity<br>↑ Lysozyme activity | (Lin et al., 2011) |
| | | | | + *Aeromonas veronii* | ↑ Survival rate | |
| Indian major carp | Oyster mushroom - derived | HK leukocytes stimulation (*in vitro*) | 1, 10 or 100 µg/ml | | ↑ Respiratory burst<br>↑ Phagocytic activity<br>↑Bactericidal activity | (Kamilya et al., 2006) |
| Indian major carp | β-1,3 glucan | Oral | 0.1% | Only β-glucan | ↑Bacterial agglutination titre<br>↑ Haemagglutination titre<br>↑ Phagocytic activity | (Sahoo and Mukherjee, 2001; Sahoo and Mukherjee, 2002) |
| | | | | + *Aeromonas hydrophila* | ↓ Mortality rate | |
| | | | | + *Edwardsiella tarda* | ↑ Antibody response<br>↓ Mortality rate | |

| Specie | β-glucan | Via | Dose | Challenged | Effects | Reference |
|---|---|---|---|---|---|---|
| Asian catfish | Yeast β-1,3 glucan | Oral | 0.1% for 7 days | Only β-glucan<br><br>+ *Aeromonas hydrophila* | ↑ Respiratory burst<br>↑Phagocytic activity<br><br>↑ Antibody response<br>↓Mortality rate | (Kumari and Sahoo, 2006a; Kumari and Sahoo, 2006b) |
| Rainbow trout | β-glucan from barley | Oral | 12.2, 16.7 or 26,4 gr/Kg | + Infectious hematopoietic necrosis virus (IHNV) | ↑ Antibody response<br>↓ Mortality rate | (Sealey et al., 2008) |
| Nile tilapia | Laminarin | Oral | 0.1% | Only β-glucan<br><br>+ *Aeromonas hydrophila* | ↑Respiratory burst<br>↑Lyzozyme activity<br>↑Bactericidal activity<br><br>↓ Mortality rate | (El-Boshy et al., 2010) |
| | β-glucan from Baker's yeast | Oral | 0.1% | Only β-glucan<br><br>+ *Aeromonas hydrophila* | ↑Respiratory burst<br>↑Phagocytic activity<br>↑Lyzozyme activity<br>↑Bactericidal activity<br>↑NO<br><br>↓ Mortality rate | |
| Sea bass | MacroGard® | Oral | 0.1% | Only β-glucan | ↑Serum complement activity 15 days post-treatment (pt)<br>↑Lyzozyme activity 30 days pt | (Bagni et al., 2000) |

### 1.6.2 β-glucan stimulation during wound healing

Beneficial effects induced by β-glucans have been described during wound healing in mammalian models. However the mechanisms involved during this process have not been well established and seem to be dependent on the type of injury treated (Chen and Seviour, 2007). Improvement of wound healing by β-glucans has been attributed to their enhancement of inflammatory responses, specifically through the induction of TNF-α production in wound macrophages. This leads to higher neutrophil infiltration to the site of injury (Roy et al., 2011) Furthermore, β-glucans have been reported to activate the production of cytokines and growth factors by macrophages of diabetic mice resulting in a faster wound closure (Berdal et al., 2007). Studies in mice infected with tetrathyridial stages of *Mesocestoides corti* showed that β-glucan can modulate liver fibrosis through the stimulation of phagocytic cells, reducing larval counts and improving liver repair (Ditteova et al., 2003). Additionally, β-glucans have shown to act as a free radical scavenger, ameliorating lipid peroxidation and providing protection against oxidative stress after spinal cord injury in rats, as well as accelerating recovery of cell damage induced in albino rats by ionizing irradiation (Kayali et al., 2005; Salama, 2011). The influence of β-glucans does not seem to be limited to immune cells. Human fibroblasts have been shown to express receptors for fungal (1,3)-β-D-glucans, triggering NF-kB nuclear binding activity and IL-6 mRNA expression upon β-glucan stimulation (Kougias et al., 2001). Furthermore, studies in cultured mouse fibroblasts have demonstrated an effect of β-glucan on cell proliferation (Son et al., 2005). Despite this observations and the wide-spread use in aquaculture (Meena et al., 2012), the effect of β-glucan during wound healing in fish has not been previously studied.

### 1.6.3 β-glucan recognition by the immune system

In mammalian models induction of cellular responses by β-glucan is associated with specific interactions with one or more PRRs present in the cellular surface or in the cytosol (Kankkunen et al., 2010). Although some of these β-glucan receptors are found in fish, their involvement in β-glucan recognition and immunomodulation has not been elucidated (Meena et al., 2012).

#### 1.6.3.1 Complement receptor 3

The complement receptor type 3 (CR3 or CD11b/CD18) has been denoted as a β-glucan receptor in mammals. It is a heterodimeric receptor composed by a β subunit (CD18), and an α subunit (CD11b), which contains a β-glucan binding site (Xia and Ross, 1999). Expression of CR3 is found in neutrophils, monocytes, NK cells and macrophages (Meena et al., 2012). Wright and co-workers showed that CR-3 mediated phagocytosis in humans upon stimulation with complement-opsonic fragments (C3b and C3bi) did not induce respiratory burst (Wright, 1983). However some years later, Cain and colleagues showed that CR-3 mediated ingestion of β-glucan in neutrophils promoted the production of oxygen radicals (Cain et al., 1987). It has been found that CR3 can facilitate antigen presentation and may control synthesis of IL-12, thereby influencing T cell responses (Meena et al., 2012). In fish, a functional study detected the presence of CR-3 complement receptor on carp head kidney macrophages, and in rainbow trout both CR-3 subunits have been cloned and characterized, although knowledge on their functional roles remains vague (Mikrou et al., 2009; Nakao et al., 2003).

#### 1.6.3.2 Dectin-1

Dectin-1 (Dentritic cell associated C-type lectin-1) is a non-classical C-type lectin which mainly binds protein ligands and is considered the main receptor for β-glucans in mammalian models. Dectin-1 is expressed predominantly by macrophages, neutrophils, dentritic cells and subpopulations of T cells (Tsoni and Brown, 2008). β-glucan recognition by Dectin-1 induces endocytosis and phagocytosis, respiratory burst responses, cytokines and chemokines, including TNF, CXCL2, IL-6, IL-10, IL-2, and IL-12. Whereas the particle uptake mediated through Dectin-1 is not dependent of Syk kinase signalling, the ROS and cytokine activity upon ligation on the other hand seems to be dependent on recruitment and activation of Syk through its cytoplasmatic hemITAM motif (Sancho and Reis e Sousa, 2012). Furthermore, it has been shown that some of these responses like pro-inflammatory cytokine production and the respiratory burst, require, or are enhanced by, cooperative signalling from MyD88-coupled TLRs (Gantner et al., 2003). To date, the presence of Dectin-1 receptor in fish remains unknown, but similar expression of immune molecules and the induction of respiratory burst have been described (see table 1)

#### 1.6.3.3 Lactosylceramide

Lactosylceramide (LacCer) is a glycosphingolipid found in the plasma membrane of many cells. It contains a hydrophobic ceramide lipid and a hydrophilic sugar component. Interaction of β-glucans with lactosylceramide receptor in human neutrophils activates the ROS production. Furthermore, alveolar epithelial cells have shown increased production of NF-κB and TNF-α after recognition of β-glucan extracted from *Pneumocystis carinii* through the LacCer receptor (Evans et al., 2005; Wang et al., 2005; Zimmerman et al., 1998). No reports of β-glucan interaction with the LacCer receptor have been made in fish.

#### 1.6.3.4 Scavenger receptors

Scavenger receptors (SRs) comprise several groups of heterogeneous molecules which are expressed by myeloid cells and some endothelial cells. Mammalian systems have shown the involvement of SRs in immunity and homeostasis. SRs can bind laminarin and other glucans, through a complex interaction which can be influenced by polymer structure, polymer charge, and other as yet undefined parameters (Rice et al., 2002)

#### 1.6.3.5 Toll-like receptors

Toll-like receptors (TLRs) are evolutionary conserved, trans-membrane proteins, which are expressed on immune cells like macrophages, dendritic cells (DCs), B cells, certain T cell subsets, and on various non-immune cells like fibroblasts and epithelial cells. Depending on the type of TLR, they can be found on the cell surface or in intracellular compartments (Kawai and Akira, 2010). TLRs are capable of sensing organisms ranging from bacteria, fungi, protozoa to viruses (Akira et al., 2006). Studies in mammalian systems have shown that several fungal PAMPs are recognized by TLR2 or TLR4 (Brown and Gordon, 2005; Gantner et al., 2003; Netea et al., 2004). Collaborative recognition of yeast has been reported for TLR2-Dectin-1 receptors leading to a strong inflammatory response (Akira et al., 2006). On the other hand, production of the anti-inflammatory cytokine IL-10 and development of Th2 responses after TLR2 activation with some fungal PAMPs have also been reported, which might be used as a fungal escape mechanism from the immune system (Agrawal et al., 2003; Netea et al., 2004). To date 17 different TLRs have been identified in different fish species (Rebl et al., 2010). Numerous studies have shown that some fish TLRs share functional properties with their mammalian counterparts. However, also remarkable differences have been found in TLR signalling cascades (Rebl et al., 2010). Hence, TLR4

which in mammals is a central receptor for the recognition of LPS is absent in most fish species, except in some cyprinids as common carp, rare minnow and zebrafish (Palti, 2011). Functional studies in zebrafish demonstrated that fish TLR4 does not recognize LPS (Sepulcre et al., 2009). Besides, all the fish genomes sequenced until now lack the co-stimulatory molecules CD14 and MD2 needed in mammalian organisms for LPS recognition (Palti, 2011; Sepulcre et al., 2009). In mammals, TLR2 has been shown to collaborate with Dectin-1 during fungal recognition, and to form dimeric combinations with TLR1 and TLR6 to recognize several bacterial pepticoglycans (PGN) and lipoproteins (Gantner et al., 2003; Kawai and Akira, 2006; Kawai and Akira, 2010). TLR2 has been identified in several fish species (Palti, 2011). Furthermore, carp TLR2 has been shown to be activated by Peptidoglycan (PGN) and Lipoteichoic acid (LTA) from *Staphylococcus aureus* (Ribeiro et al., 2010). But the presence of dectin-1 receptor is unknown, and the formation of heterodimers with TLR1 or TLR6 is still unclear.

## 1.7 Methodological features of the thesis

### 1.7.1 The damage model *in vivo*

Mechanically induced tissue damage has been previously used in order to mimic inflammatory responses to ectoparasites in carp (Gonzalez et al., 2007). Furthermore, the methodology was also used to investigate local responses in muscular tissue after injury in rainbow trout (Ingerslev et al., 2010a). During the work of this thesis, the mechanical tissue damage model is use in order to analyse the modulatory effect of β-glucans during wound healing processes of carp.

After sedation of the carps, 5mm biopsy punches were used to inflict the tissue damage in skin and muscle. The injury was always made in the left side of the fish, above the lateral line (Fig 2A). The subsequent samplings were performed using 8mm biopsy punches to ensure sampling of the damaged area plus the neighbouring tissues. Furthermore, a sample from the non-damaged side was taken as an internal control, allowing the study of local tissue responses in individual fish (Fig 2B). Images were made during each sampling point in order to follow wound closure (Fig 2C).

Fish handling and experimental procedures were performed according to the Danish legislation and the FELASA (Federation of Laboratory Animal Science Associations) ethical guidelines.

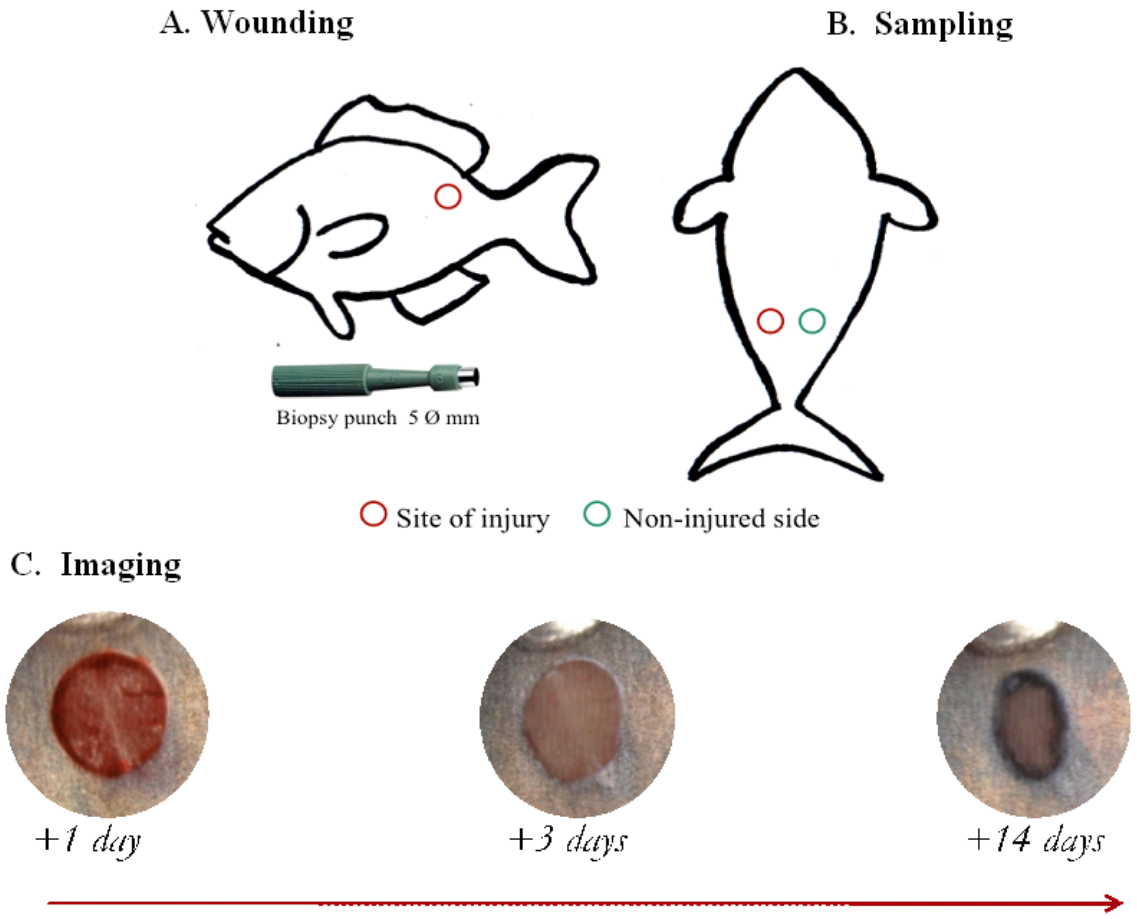


**Figure 2. Mechanically induced tissue damage scheme.** (Paper II) **A)** 5mm biopsy punches were used to inflict tissue damage, the injury was performed on the left side of the fish above the lateral line. **B)** 8 mm biopsy punches were used to take a sample from the injury (left side, red circle) and an internal control (right side, green circle). **C)** Imaging of the wound healing process.

### 1.7.1.1 Immune molecules studied during wound healing *in vivo*

- **Interleukin-1β (IL-1β)**

IL-1β is a pro-inflammatory cytokine and central component of the inflammatory response (Dinarello, 1998). IL-1β has been shown to be up-regulated in response to pathogens and to promote different stages during sterile inflammation (Ingerslev et al., 2010a; Lindenstrøm et al., 2003; Rider et al., 2011) IL-1β activation of immune cells and

resident cell types during infection and injury has also been reported, leading to the expression of other cytokines and growth factors, production of reactive oxygen species and enhanced leukocyte migration (Martin and Leibovich, 2005; Werner and Grose, 2003). IL1-β is recognized by two receptors IL-1R1 and IL-1R2, due to the powerful inflammatory nature of IL-1β, IL-R2 acts as a decoy receptor inhibiting the action of IL-1β, and avoiding detrimental consequences of its powerful inflammatory nature (Lindenstrøm et al., 2003).

- **Interleukin-6 (IL-6)**

Mammalian models have shown that members of the IL-6 cytokine family are involved in numerous physiological processes like cellular development, inflammatory function and immune responses (Hwang et al., 2007). The importance of IL-6 during wound healing was demonstrated with studies in IL-6 knock-out mice, where wound healing took up to three times longer due to a delay in re-epithelization (Gallucci et al., 2000). However, excess of IL-6 during wound healing processes is associated with cutaneous scarring (Werner and Grose, 2003). Fujiki and co-workers described a common carp cytokine-like cDNA inducible by inflammatory stimulation. They related this molecule, termed M17, to the IL-6 cytokine family (Fujiki et al., 2003). In Japanese flounder, a M17 homologue was also cloned and characterized, it was observed to be highly expressed in immune-related tissues and to be induced by LPS, polyI:C and PGN *in vitro*, suggesting its involvement in fish immunity (Hwang et al., 2007). Furthermore, goldfish M17 was found to induce the production of nitric oxide in goldfish macrophages and to facilitate the differentiation of sorted monocytes into macrophages (Hanington and Belosevic, 2007).

- **Interleukin-8 (IL-8)**

IL-8 is a member of the CXC chemokines family and it is known as the major bioactive chemoattractant for neutrophils in humans (Werner and Grose, 2003). High levels of IL-8 have been associated with impair wound repair. However studies with human grafts in chimeric mice demonstrated that topical application of IL-8 stimulated re-epithelialization (Werner and Grose, 2003). In rainbow trout, IL-8 has been shown to be up-regulated following mechanical tissue damage and infection with *Moritella viscosus* (Ingerslev et al., 2010a). Furthermore, high up-regulation of IL-8 has been measured after DAMP stimulation of rainbow trout fibroblast *in vitro* (Ingerslev et al., 2010b).

- **Mucin 5b (Muc5b)**

Mucins are glycoproteins synthesized by mucosa or skin. Most secreted mucins are synthesized in goblet cells of epithelial surfaces or mucous cells of exocrine glands, and they have different function such as lubrication, chemical barriers and protection against pathogens. In humans, the *MUC5B* gene has been found to be expressed mainly in the mucous glands of the respiratory sytem, salivatory glands, pancreas and cervix (Roussel and Delmotte, 2004). In carp *Muc5B* is highly expressed in skin and gills, and at a lesser degree in brain and liver (van der Marel et al., 2012).

### 1.7.2 *In vitro* studies

Cellular model systems have been important contributors for much of the biomedical, physiological and toxicological research during the past years. Cell lines and cells isolation have been extensively used to elucidate the biochemical and physiological mechanisms by which the organism reacts to disease, wounds and environmental threats (Hightower and Renfro, 1988; Lakra et al., 2011). *In vitro* studies allows the researcher to investigate cellular functioning under controlled and specific conditions, thereby eliminating parameters that could mask the responses of interest for the study. However, the organism as a whole should also be taken into account during the processes investigated. During this thesis, fibroblasts, leukocytes and reactive oxygen species were chosen to investigate their involvement in wound healing, and to elucidate the effector mechanisms of β-glucan during tissue repair. The main components for the *in vitro* studies of this thesis are summarized below.

#### 1.7.2.1 Fibroblasts

Fibroblasts are the main producers of collagen and other extracellular matrix (ECM) components, generating and maintaining the tissue supporting framework (Mescher and Neff, 2005). After injury, the inflammatory response is followed by attraction and proliferation of fibroblast, becoming the predominant cell type in the wound site (Diegelmann and Evans, 2004). Fibroblasts are considered one of the most important cell types involved in tissue repair. They have shown phagocytic activities, production of cytokines, chemokines, and growth factors, and are recognized as major regulators of inflammation during wound healing (Arlein et al., 1998; Diegelmann and Evans, 2004; Trengove et al., 1999). Furthermore, fibroblasts are considered a link between the immune system and the tissue repair machinery, a key tool driving the fate of wound healing towards complete regeneration, tissue repair,

fibrosis or chronic wound (Silzle et al., 2004; Wallach-Dayan et al., 2007). In fish, the phagocytic activities of fibroblasts have been described after skin wounding in carp (Iger and Abraham, 1990). Additionally, *in vitro* studies on rainbow trout derived fibroblasts, showed the expression of immune genes such as *IL-1β, IL-8, IL-10, TLR3* and *TLR-9* after stimulation with DAMPs (Ingerslev et al., 2010b).

#### 1.7.2.2 Macrophages

Macrophages originate from the myeloid lineage and are resident in almost all tissues, where they are essential to maintain tissue homeostasis. Macrophages are equipped with a broad range of PRRs that make them remarkable phagocytic cells (Geissmann et al., 2010). Macrophages respond rapidly to infection by engulfing and destroying pathogens in intracellular vesicles through the production highly toxic compounds like ROS and NO. Furthermore, they can act as antigen presenting cells, linking the innate and adaptive immune responses (Mosser and Edwards, 2008). Macrophages play an important role during tissue repair, due to their involvement in inflammation, their phagocytic activity. Furthermore, the production of numerous cytokines and growth factors by macrophages can regulate fibroblast proliferation and angiogenesis (Martin and Leibovich, 2005; Medzhitov, 2008).

#### 1.7.2.3 Reactive oxygen species (ROS)

The production of ROS, also known as respiratory burst, is one of the earliest cellular responses following pathogen recognition. Its initiation is marked by an increase in oxygen cellular uptake, followed by the one electron reduction of molecular oxygen ($O_2$) to superoxide anions ($O_2^-$). This reaction is catalysed by the membrane-associated enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, using NADPH as the electron donor (Babior, 1999; Bellavite, 1988; Chung and Secombes, 1988; Madamanchi and Runge, 2007; Pick and Mizel, 1981). Further reduction of $O_2^-$ produces hydrogen peroxide ($H_2O_2$), which occurs either as a spontaneous dismutation, especially at low pH, or as a catalyzed reaction by a family of enzymes called Superoxide dismutase (SOD). Additional reactions of $O_2^-$ and $H_2O_2$ may lead to the formation of hydroxyl radicals ($OH^-$), especially in the presence of iron through the Fenton or Haber-Weiss reactions. The interaction of $H_2O_2$ with myeloperoxidase (MPO) can produce hypochlorous acid and other toxic metabolites if $H_2O_2$ is not dismutated to water and molecular oxygen by the enzyme catalase which can act as a natural scavenger (Arockiaraj et al., 2012; Babior, 1999; Fridovich, 1978; Genestra, 2007; Jones, 1982; Lushchak, 2008). A schematic representation with main products of the

respiratory burst is shown in figure 3. Besides the significant function of ROS during infection clearance, their importance during wound healing processes has been established. ROS have been shown to be involved in cell proliferation, but can also caused detrimental effect on host tissues due to oxidative stress (Kanta, 2011). Mammalian studies have shown that an extraordinary coordination for the quantities and timing in the production of ROS is needed, and exerts an important impact in the outcome of wound healing (Diegelmann and Evans, 2004; Wallach-Dayan et al., 2007). For example, ROS in low concentrations (1-25μM) function as second messengers and promote cell proliferation, at higher concentrations (25-50μM) have microbicidal effects, and in excessive amounts (>50μM) they can impair cell growth and cause apoptosis (Kanta, 2011). Furthermore, although it is conceivable that the majority of the ROS are produced by immune cells during the inflammatory phase, fibroblast and other cell types can contribute to their production at a lesser extent during wound healing process (Kanta, 2011; Witte and Barbul, 2002).

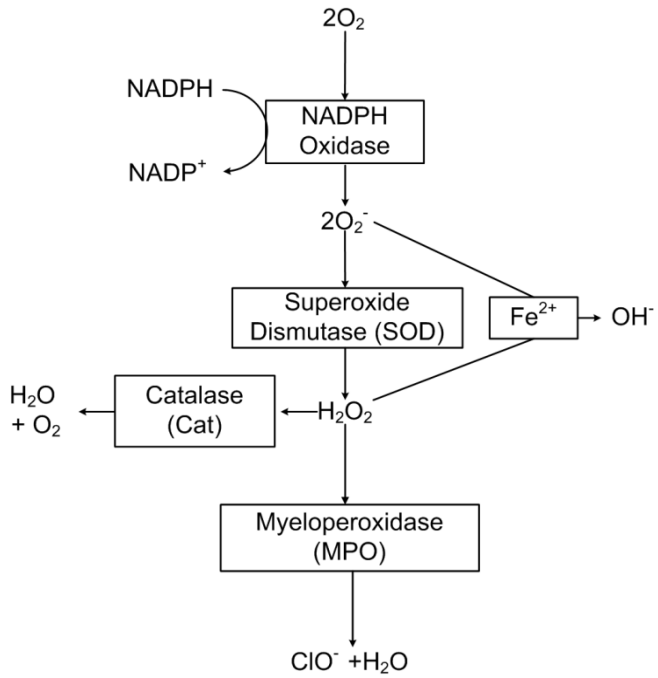


**Figure 3 Schematic representation of the Respiratory burst main products**. The membrane-associated enzyme nicotinamide adenine dinucleotide phosphate (NADPH) catalyzes the reduction of molecular oxygen ($O_2$) to superoxide anion ($O_2^-$), using NADPH as the electron donor. Further reduction of oxygen produces hydrogen peroxide ($H_2O_2$), which occurs either as a spontaneous dismutation, or as a catalyzed reaction by a family of enzymes called Superoxide dismutase (SOD). Additional reactions of $O_2^-$ and $H_2O_2$ may lead to the formation of hydroxyl radicals ($OH^-$), especially in the presence of iron through the Fenton or Haber-Weiss reactions. The interaction of $H_2O_2$ with the enzyme myeloperoxidase (MPO) can produce hypochlorous acid and other toxic metabolites, or $H_2O_2$ is dismutated to water and molecular oxygen by the enzyme catalase.

### 1.7.2.4The scratch-wound assay

Scratch-wounding of cell cultures has been previously used in order to study re-epithelialization processes in mice (Matsubayashi et al., 2011). During this thesis, the scratch-wound assay was used in order to follow fibroblast wound recovery dynamics. Furthermore, the direct effect of hydrogen peroxide, DAMPs and β-glucans during wound recovery was analyzed.

To perform this assay fish fibroblast were seeded in 6-well culture plates, and linear-scratches were inflicted using a pipette tip as shown in figure 4A. The objective of the scratch was to cause cell death in specific areas of the culture. Figure 4B shows the damage inflicted by the scratch wound assay in a monolayer stained with fluorescein diacetate and propidium ioide two hours after wounding. Two perpendicular scratches are visible (black area), live cells are stained in green, while nonviable cells fluoresced red. Once the scratch wound methodology was established, different modulators were added to the wells and the wound recovery was followed daily using microscopy pictures and computational tools (see figure 4C).

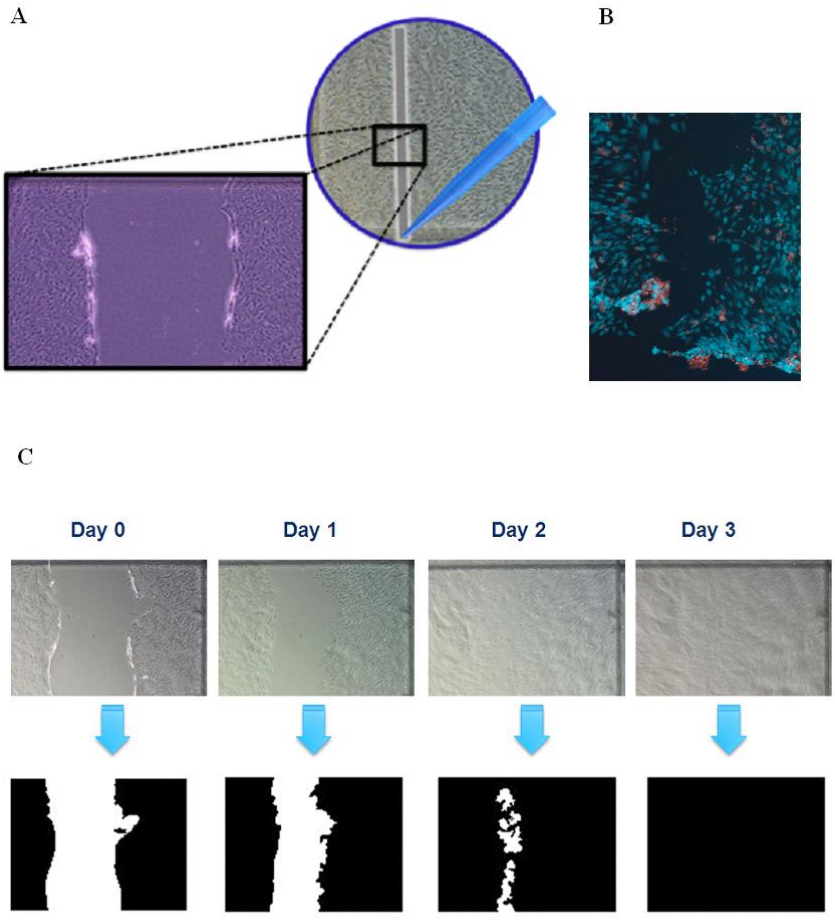


**Figure 4 The Scratch-wound assay. A)** Monolayers of fish fibroblast are wounded with a pipette tip. **B)** Two perpendicular scratches are visible (black area) in a section of a wounded culture. Staining with fluorescein diacetate and propidium ioide allows visualization live cells (green color) and nonviable cells (red color). **C)** Scratch-wounded cultures were followed with pictures daily, and the images were analyzed with computational tools.

## 1.8. Aims of the PhD study

The overall aim during this PhD was to study the modulatory effects of β-glucan during wound healing in carp, and to explore what mechanisms, molecules and cell types were involved during the modulation.

Firstly, an assessment of methodologies used to measure reactive oxygen species, which have shown great importance during infection, immune-modulation and wound healing processes in mammalian systems, was made.

Further, the immune modulatory effect of β-glucan during skin wound healing in carp was tested *in vivo*.

Lastly, *in vitro* experiments were designed to test the response of macrophages and fibroblast to mechanical injure and β-glucans. Two questions raised during this process were: Can carp macrophages differentiate between self (DAMPs) and non-self (PAMPs)? And can DAMPs and β-glucan directly stimulate fibroblast during wound healing?

## 2. Discussion:

Wound closure and tissue repair are very important to keep health integrity of any organism (Martin, 1997; Singer and Clark, 1999). In fish, skin integrity is important to protect them from pathogens present in the aquatic environments, which would rapidly colonize an open wound. Furthermore, fish skin is also important in homeostasis, osmoregulation and lubrication for swimming efficiency (Gadomski et al., 1994; Noga, 2000). Infections, high stocking densities, fish handling, grading and marking, human borne pollutants in the aquatic systems and even environmental factors can damage fish tissues (Bouck and Smith, 1979; Ellis et al., 2002; Jeney and Jeney, 1995; Johnson, 2004). However, the multiple cellular and molecular processes intended to restore tissue integrity and complete wound healing in fish are limited and not well understood.

The immune stimulatory effects of β-glucan have been studied in healthy and infected fish, showing their capability to boost the fish immune system and increase resistance to different pathogens (see table 1). However, the effect of β-glucan during wound healing in fish has not been previously studied. Hence, this thesis focuses in wound healing processes in common carp stimulated with β-glucan, and discusses some of the possible cellular and molecular mechanisms underlying β-glucan immune stimulation during wound closure in carp.

### 2.1 A matter of method

What is measured and when is it measured? The parameters studied during a scientific research give to the researcher tools to understand the processes involved in their matter of concern. Thus, the choice of the methodologies used to measure such parameters should not be taken lightly.

The production of reactive oxygen species (ROS) is associated with infection clearance (Chung and Secombes, 1988). It is modulated after β-glucan stimulation (see table 1), and it has shown to be an important chemoattractant during wound detection in fish (Niethammer et al., 2009). Therefore ROS production was an interesting parameter to study during this thesis. Several methodologies for the study of ROS production have been developed through the years, some of the most important are summarized in the introduction of paper I.

A comparison between the nitroblue tetrazolium assay (NBT) and the real-time luminol-enhanced chemiluminescence assay (RT- luminol assay) is the main subject of paper I. NBT is perhaps the most popular methodology for monitoring radical production (Weber, 1990). And the RT-luminol assay is a protocol based in the native chemiluminescence of the phagocytes (Allen et al., 1972). After stimulation of total head kidney cells and freshly isolated head kidney leukocytes with two different β-glucans (Zymosan and MacroGard®), both methodologies detected the production of oxygen radicals, and allowed the detection of dose-related changes on the radical production magnitude (paper I). Thus, both methods can be used to evaluate the effect of immune-stimulants, vaccines and pharmacological agents on the immune system, and as a general indicator of fish health status, despite the degree of cell sorting (John et al., 2002; Kamilya et al., 2006; Verho et al., 2005). However, co-stimulation of total head kidney cells with the oxygen radical scavengers superoxide dismutase (SOD) and catalase (Jones, 1982; McCord and Fridovich, 1969), revealed that NBT measured only superoxide anion ($O_2^-$), while RT-luminol assay measured $O_2^-$, hydrogen peroxide and related oxygen radicals such as hypochlorous acid and hydroxyl radical (Figure 5). Furthermore, since the NBT is based in the intracellular reduction of the tetrazolium salt to formazan (Baehner and Nathan, 1968), it requires the cell lysis for the completion of the measurement. Therefore, it is a one-point measurement, and leaves aside the possibility of examine the reaction kinetics. On the other hand, the RT-luminol assay allowed the measurement of the oxygen radicals production at any given moment. The importance of such differences was confirmed in Paper III, where the RT-luminol assay allowed the detection of differences on kinetic patterns and type of oxygen radicals produced after carp head kidney derived leukocytes stimulation with DAMPs and PAMPs. Furthermore, the amount of cells required for the experiments and the sample manipulation (therefore the risk of pipetting mistakes) is reduced with the RT-luminol assay in comparison to the NBT.

In conclusion, NBT and RT luminol assay detect oxygen radical production from carp head kidney leukocytes. The detection of ROS with both methodologies was successful in mixed cell types suspensions (total head kidney cells) and in more cell type defined suspensions (head kidney leukocytes). Therefore, both methods can be used as an indicator of the general health status in fish. However, only the RT-luminol assay allows the detection of hydrogen peroxide production and the measurement of oxygen radical kinetics, important parameters to study during wound healing processes.

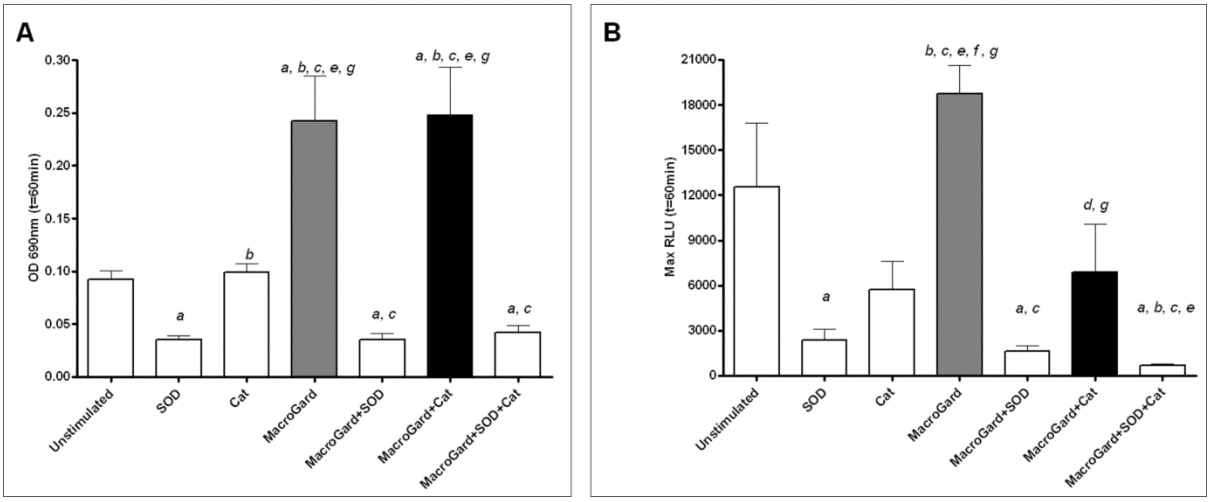


**Figure 5. Identification of the oxygen radicals measured by NBT and RT-luminol assay.** Comparison of the nitro blue tetrazolium NBT assay (A) and the Real-time luminol assay (B) in relation to the type of oxygen radicals being produce after stimulation of t-HK cells with MacroGard® (100µg/ml) and co-stimulation with SOD (250 U/ml) or catalase (300U/ml). The plots show the mean value of four independent studies for NBT and five for RT-luminol assays. Error bars represent standard error of the mean, *a*= significant difference to unstimulated sample, *b*= significant difference to SOD, *c*= significant difference to Cat, *d*= significant difference to MacroGard®, *e*= significant difference to MacroGard® +SOD, *f* = significant difference to MacroGard® +Cat, *g*= significant difference to MacroGard® +SOD +Cat . All the difference presented a P value <0.05. (Paper I)

## 2.2 Can tissue repair in carp be modulated by β-glucans?

For the first time, a scientific project has evaluated the effects of β-glucan treatment in wound closure of fish (paper II). During this study, application of MacroGard® and 6.3 kDa β-glucan bath treatments to mechanically wounded carps resulted in a faster wound closure response (Fig 6). Studies in mammalian systems have described improved wound healing after treatment with β-glucans, but the mechanisms involved during β-glucan stimulation of wound healing processes have not been entirely established (Cerci et al., 2008; Karaaslan et al., 2012; Petravic-Tominac et al., 2010). Different studies suggest that β-glucan enhances macrophage function during the inflammatory phase, through the induction of cytokine production and enhancement of phagocytic activity (Browder et al., 1988; Roy et al., 2011; Sakurai et al., 1992). Furthermore, β-glucan-stimulated macrophages have shown to interact with fibroblasts and enhance their migration and collagen synthesis function, and a direct effect of β-glucan on fibroblast proliferation has also been observed (Portera et al., 1997; Son et al., 2005; Son et al., 2007).

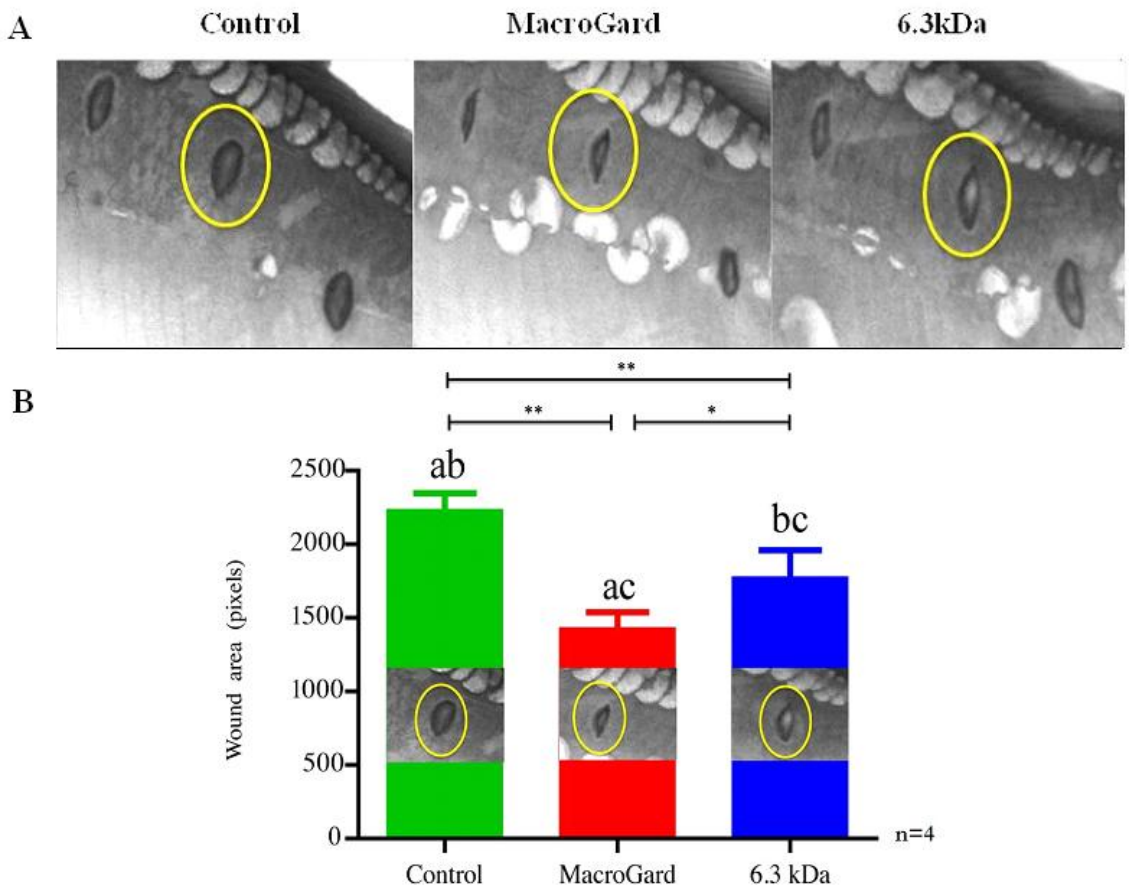


**Figure 6 Wound closure in carp stimulated with β-glucans.** After sedation, carps were wounded with a 5mm biopsy punch. Fish were kept for two weeks in tap water (control), MacroGard® bath (0.1 µl/ml) or 6.3 kDa β-glucan bath (0.1 µl/ml). Images were taken from each fish and processed with image analysis computer tools to follow wound closure. **A)** Images of the wound area in carp 14 days post-wounding, taken with VideometerLab. from right to left: Control carp, Carp stimulated with MacroGard® bath, and carp stimulated with 6.3kDa β-glucan bath. The yellow circles highlight the central wound of each fish. **B)** Statistical representation of the wound sizes 14 days post wounding. Bars represent the mean value of wound pixels and error bars show standard deviation, n=4 except by MacroGard® where n=3. *=$P$<0.05, **=$P$<0.01

In paper II, the expression of the immune molecules IL-1β, IL-6 and IL-8 seemed to increase in all mechanically wounded carps. Previous studies in experimental wounded rainbow trout, and in DAMP (rainbow-trout fibroblast lysates) stimulated rainbow trout fibroblast cultures, also showed an up-regulation in the expression of IL-1β and IL-8 (Ingerslev et al., 2010a; Ingerslev et al., 2010b; Ingerslev et al., 2010b). In addition, a statistically significant increase in the expression of the pro-inflammatory cytokines IL-1β and IL-6 at day 1 and 3 for the mechanically wounded carps treated with β-glucans was measured (see table 2). The production of these cytokines by immune cells and resident cell types during injury in mammalian systems, have been shown of great importance during wound healing response

(Grellner et al., 2000; Werner and Grose, 2003). They are suggested to enhance the expression of growth factors in the wound area (Hu et al., 2010b; Martin and Leibovich, 2005; Werner and Grose, 2003), and induce the production of reactive oxygen species (Kanta, 2011; Murrell et al., 1990; Niethammer et al., 2009). Therefore, the β-glucan induced up-regulation of IL-1β and IL-6 in the wound area might positively influence the wound closure described in paper II. On the other hand, an increased expression of the chemokine IL-8 was measured at day 3 and 14 in untreated mechanically wounded carps (see table 2). IL-8 is considered the major bioactive chemoattractant for neutrophils in humans, promoting their migration and infiltration to the wound site. IL-8 expression is needed during the first stages of wound healing, but excessive neutrophil infiltration can be a cause of delayed wound healing and chronic ulcers (Werner and Grose, 2003). The late expression of IL-8 in untreated carps could therefore sustain the influx of neutrophils even two weeks after injury, delaying wound closure.

The protective function of mucins, one of the main components in the mucus, is found to be conserved from shark to mammals (Theodosiou et al., 2007). In carp, *muc5b* is highly expressed in skin and gills, and has been shown to increase its expression after β-glucan feeding (van der Marel et al., 2012). In wounded carps, the expression of *muc5b* showed a minor increase, but it did not appear to be influenced by the β-glucan bath treatment (see table 2). The producers of mucus are goblet cells (Shephard, 1994). They originate adjacent to the basal membrane and maturate while ascending to the surface of the epidermis (Henrikson and Gedeon-Matoltsy, 1967a). The wounding procedure employed during this experiment removed all the skin and upper muscle layer. Therefore, goblet cells were most likely absent in the sampling sites, which could account for the unsubstantial expression of *muc5b*. Therefore, for further studies it is recommended to sample and analyze the expression of *muc5b* in bordering positions to the wound.

During this study, the use of internal controls in non-damaged sites showed that the up-regulation of the studied genes was localized to the wound area. Furthermore, the absence of marked differences in the production of oxygen radicals by head kidney leukocytes among the different treatments supports that β-glucan stimulation during the wound healing process does not elicit a systemic response, instead it is localized in the site of injury (paper II).

**Table 2. Gene expression profiles of experimentally wounded carps stimulated with MacroGard® and 6.3kDa β-glucan.** After sedation, 5mm biopsy punches were used to inflict the tissue damage in the skin of carps, fish were kept for two weeks in tap water (control), MacroGard® bath (0.1 µl/ml) or 6.3 kDa β-glucan bath (0.1 µl/ml). Samplings were performed using 8mm biopsy punches at day 1, 3 and 14 post wounding. Expression profiles of IL-1β, IL-6, IL-8 and Muc5b were measured with quantitative real-time PCR. Purple triangles represent fold up-regulation relative to the non-wounded internal control, ND= non-detected. *= P< 0.05, **= P< 0.01. (n=3)

| 1-5 | CARP SKIN | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 5-25 | Untreated | | | MacroGard® | | | 6.3 kDa | | |
| >25 | 1d | 3d | 14d | 1d | 3d | 14d | 1d | 3d | 14d |
| IL-1β | N.D | | | | * | | * | | |
| IL-6 | N.D | | | | * | | | | |
| IL-8 | | * * | * * | | | | | | |
| Muc5b | | | * | | | | | * | |

The occurrence of a possible direct β-glucan effect on fibroblast proliferation during wound healing was evaluated using an image based *in vitro* assay. Scratch wounded CCB fibroblasts cultures were stimulated with two different β-glucans (MacroGard® and Zymosan) and the wound closure was followed (paper III). Direct β-glucan stimulation of the scratch-wounded fibroblast cultures did not result in enhanced cell proliferation. Therefore, it suggests that the interaction between fibroblasts and immune-cells is needed for the β-glucan enhancement of wound closure in carp observed in paper II.

Summarizing, β-glucan bath treatment promoted wound closure in carp. This is could be due to the enhancement of an early inflammatory response, but with the prompt withdraw of an elevated influx of neutrophils. Furthermore, the effect of β-glucan seems to be orchestrated by the immune cells, since no direct effect of β-glucan on fibroblast proliferation was observed, and it appears to be a localized immune response of the wound site.

### 2.3 Carp head kidney derived macrophages can differentiate between PAMPs (non-self) and DAMPs (self)

Mammalian systems have illustrated that recognition of damage, either due to infection or "sterile" cell death (mechanical damage), is associated with immune recognition of PAMPs

and DAMPs (Bianchi, 2007; Hansen et al., 2011; Rock et al., 2010; Schreml et al., 2010). In fish, the innate immune response to infection and PAMP recognition has been relatively well characterized (Alvarez-Pellitero, 2008; Chen et al., 2012; Ribeiro et al., 2010; Sepulcre et al., 2007; Zou et al., 2010). However, the situation about fish immune response to sterile tissue damage and DAMP recognition is not so clear. Ingerslev and co-workers demonstrated that rainbow trout fibroblasts cultures (RTHDF cell line) expressed an up-regulation of immune relevant genes upon stimulation with RTHDF cell lysates (debris and supernatants) (Ingerslev et al., 2010b). Furthermore, an *in vitro* study of gilthead seabream head kidney leukocytes indicated that collagen fragments, produced by the action of different host proteases, were sensed by the fish phagocytes and induced the production of oxygen radicals (Castillo-Briceño et al., 2009). Since ROS are believed to play an important role in both scenarios (infection and "sterile" tissue damage) (Burdon et al., 1996; Chung and Secombes, 1988; Kanta, 2011; Lundén et al., 2002), it was interesting to test if they could act as messengers, driving cellular responses towards pathogen eradication or tissue repair.

Paper III demonstrates that head kidney derived (HK) leukocytes can differentiate between PAMPs and DAMPs. After PAMP (β-glucan) and DAMP (Fibroblast lysates) stimulation, HK leukocytes displayed different patterns of ROS production. PAMP stimulation resulted in a strong and fast oxygen radical production response (fig 7A) dominated mainly by superoxide anion (fig 7B). This is consistent with the clearance of infection as described for pathogens like *Vibrio anguillarum* (Stave et al., 1985), *Yersinia ruckeri* (Stave et al., 1987), *Aeromonas hydrophila* (Chen and Dexiang, 1991) and *Edwarsiella ictaluri* (Waterstrat et al., 1991). In these cases, the main purpose is to eradicate the pathogen before it reproduces and affects the host in a greater manner. On the other hand, DAMP stimulation led to a mild, more controlled and long lasting oxygen radical production response (fig 7A). It was dominated mainly by hydrogen peroxide (fig 7B), and consistent with a tissue repair response. Niethammer and co-workers elegantly demonstrated that a gradient of hydrogen peroxide can mediate wound healing after tail-fin incision in zebrafish. The hydrogen peroxide gradient attracts leukocytes and recruits them to the site of injury (Niethammer et al., 2009). Additionally, mammalian models have suggested the role of oxygen radicals as cellular messengers and stimuli for cellular proliferation when administered in low and continues doses, especially for hydrogen peroxide (Burdon, 1995; Burdon et al., 1996; Mander et al., 2006). To test if hydrogen peroxide constituted a stimuli for wound closure, the scratch wound assay was used. CCB fibroblast wounded cultures were stimulated daily

with hydrogen peroxide and the wound closure was followed based on images (Paper III). Stimulation with low doses of $H_2O_2$ (5 and 10µM) seemed to improve wound recovery during the whole trial period, but stimulation with 300µM of $H_2O_2$ impaired fibroblast growth and cause cell death (figure 8). These results support the idea that in the case of a sterile wound, the lack of an infection threat would make the organism to focus in the regeneration of the tissues. The production of low and steady amounts of hydrogen peroxide might act as neutrophil attractant and at the same time stimulate resident cell proliferation. Fish would then avoid the production of high amounts of oxygen radicals such as superoxide anion, which are useful to eradicate pathogens, but that can be detrimental to the host causing oxidative stress and tissue damage (Betteridge, 2000; Le Bras et al., 2005).

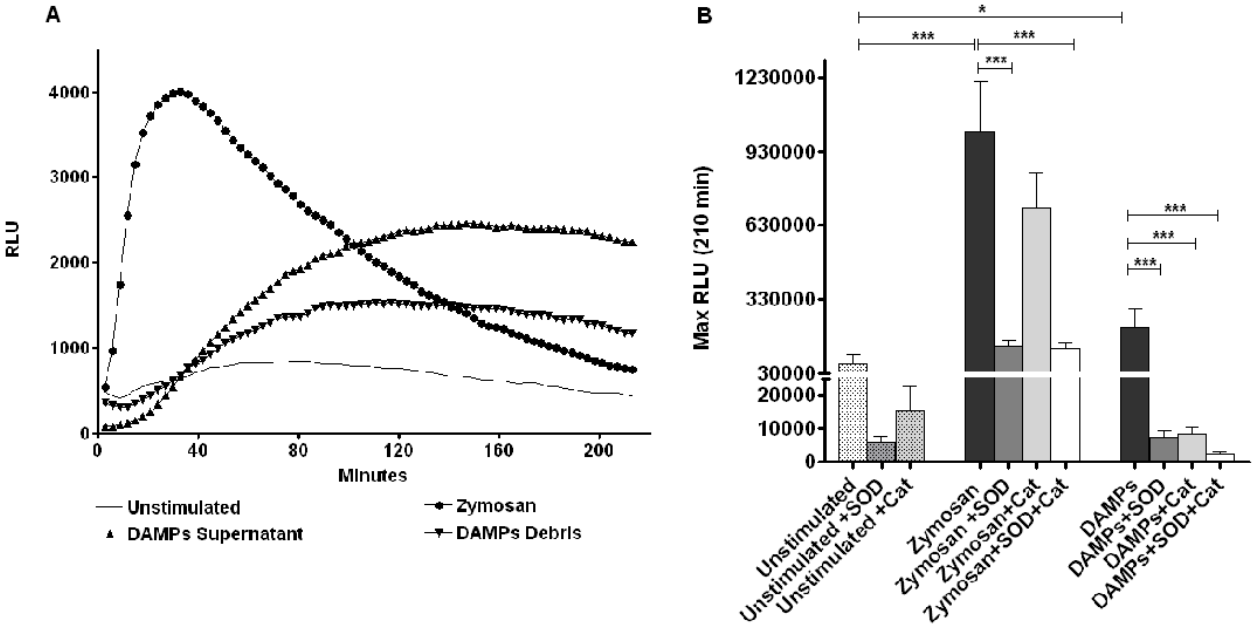


**Figure 7. Carp head kidney derived leukocytes display different oxygen radical production kinetics after PAMP and DAMP stimulation. A)** Carp head kidney derived leukocytes were stimulated with 100µg/ml of Zymosan, DAMPs supernatant from 0.5X106 CCB fibroblasts, DAMPs debris from 0.5X106 CCB fibroblasts or left untreated. Oxygen radical response was measured by RT-luminol assay during 210 minutes. The graph shows the mean value of the oxygen radical response kinetics (n=6, except for Zymosan where n=5). **B)** Carp head kidney derived leukocytes were stimulated with 100µg/ml of Zymosan, DAMPs supernatant from 0.5X106 CCB fibroblasts or left untreated. To identify the type of reactive oxygen species produced after stimulation of Zymosan and DAMPs, the oxygen scavengers superoxide dismutase (SOD, 250 U/ml) and catalase (Cat, 300 U/ml) were used. The Oxygen radical response was measured by RT-luminol assay, the graph shows the mean values of the integral of RLU (Max RLU) recorded by the luminometer between 0 and 210 minutes. Error bars represent standard error of the mean, n=8 except for Zymosan+SOD, Zymosan+Cat and Zymosan+SOD+Cat where n=6. Data was statistically analyzed using Mann Withney test. * = P< 0.5, *** = P< 0.001.

In conclusion, carp HK derived macrophages can recognize PAMPs and DAMPs, and in response display different patterns of oxygen radical production. Therefore, ROS patterns might be one of the possible ways in which fish alert the immune system and drive the immune response to cope with the threat they face.

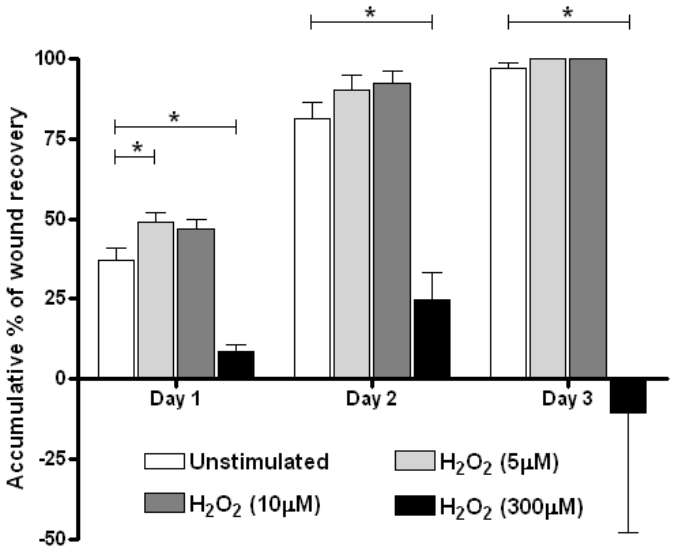


**Figure 8 Modulation of wound recovery by CCB fibroblast with hydrogen peroxide**. Monolayers of CCB fibroblasts were scratches-wounded (≈700 µm) and stimulated with 5, 10, 300 µM Hydrogen peroxide, or left untreated. The graph shows the mean values of the wound recovery percentage, the error bars represent the standard error of the mean, * = P< 0.5. n=6 except for H2O2 (300µM) where n=3.

# 3 Supplementary data and discussion

The data discussed in this section was obtained during the period of the PhD study in close collaboration with the cellular biology and immunology group (CBI) from Wageningen University. Since it is an ongoing research it has not been included in any of the accompanying papers. However, we consider important to highlight the possible involvement of some of the measured parameters from β-glucan stimulated carp macrophages during wound healing processes.

## 3.1 Possible involvement of Nitric oxide (NO) on β-glucan induced immune modulation of carp wound healing.

NO is produced by leukocytes during the initial stages of the inflammatory response and has been proposed as a modulator of the immune response involved in pathogen eradication in mammalian systems and fish (Rieger and Barreda, 2011; Wink et al., 2011). In addition,

some scientific reviews based in mammalian models have described the importance of NO during wound healing (Soneja et al., 2005; Witte and Barbul, 2002), while others focus more on the detrimental effects of the same molecule on cell proliferation (Villalobo, 2006). NO is formed by the oxidation of L-arginine to L-citrulline by NO synthase (NOS) (Wink et al., 2011).Three forms of NOS have been identified in mammals: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS), from which only iNOS has been shown to date to be involved in immune defense (Knowles and Moncada, 1994). Mammalian fibroblasts have been shown to produce NO following tissue injury (Schaffer et al., 1997). Furthermore, dermal fibroblasts from iNOS knock-out mice showed reduced cell proliferation, impaired collagen synthesis and decreased contractile properties (Shi et al., 2003). The direct involvement of NO during wound healing in fish has not been studied.

During this study, *in vitro* stimulation of HK derived macrophages with β-glucan induced the production of NO in a dose-related manner ranging between 18.5 and 31.69 μM Nitrate (see figure 9). Furthermore, iNOS expression was up-regulated in HK derived macrophages after 6 hours stimulation with β-glucan (see figure 10). Shi and co-workers reported that addition of 5 to 25 μM exogenous NO to iNOS knock-out murine fibroblasts restored collagen synthesis, therefore improving wound healing, while 50-400μM NO was detrimental for collagen production. The amount of NO released in mechanically wounded carps during β-glucan bath treatment was not measured. However, mature macrophages in the wound could probably produce NO upon β-glucan stimulation in a similar manner than the head kidney macrophages tested *in vitro*, stimulating collagen synthesis. Still, more research on this topic is needed. The measurement of NO levels in the wound site and the iNOS gene expression profile of wound resident cells would help to corroborate this hypothesis.

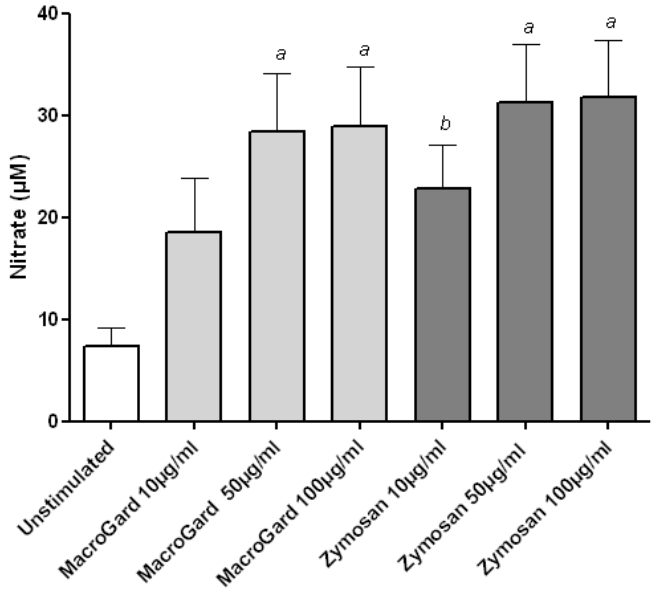


**Figure 9 Production of NO by head kidney phagocytes as measured by the detection of nitrite after 24 hours.** Head kidney derived macrophages were seeded in 96 well plates at 0.5x106 cells/well and stimulated with 10, 50 or 100 µg/ml of MacroGard®or Zymosan. Nitric oxide content was measured by Griess reaction in cell culture supernatants. Concentration of nitrite in µM was determined using a sodium nitrite standard curve. (at least) n=3 fish. Bars show the average + SD for n=3 fish. The graph shows the mean values of at least 3 independent experiment, the error bars represent the standard error of the mean. *a* = different to the unstimulated cells at P< 0.01, *b* = different to the unstimulated cells at P< 0.5.

## 3.2 Cytokine gene expression of β-glucan stimulated head kidney derived macrophages

Cytokines secreted by the cells of the immune system mediate diverse cellular responses in immunity and inflammation (Hanington et al., 2009). In addition, the complex and coordinated integration of cytokines, chemokines and growth factors, are directly related to the success of wound healing processes (Madhyastha et al., 2012). During this study, cytokine responses of β-glucan stimulated HK derived macrophages were measured with real-time quantitative PCR relative to the house-keeping gene 40S (see figure 10). The possible involvement in wound healing of the measured cytokines is discussed.

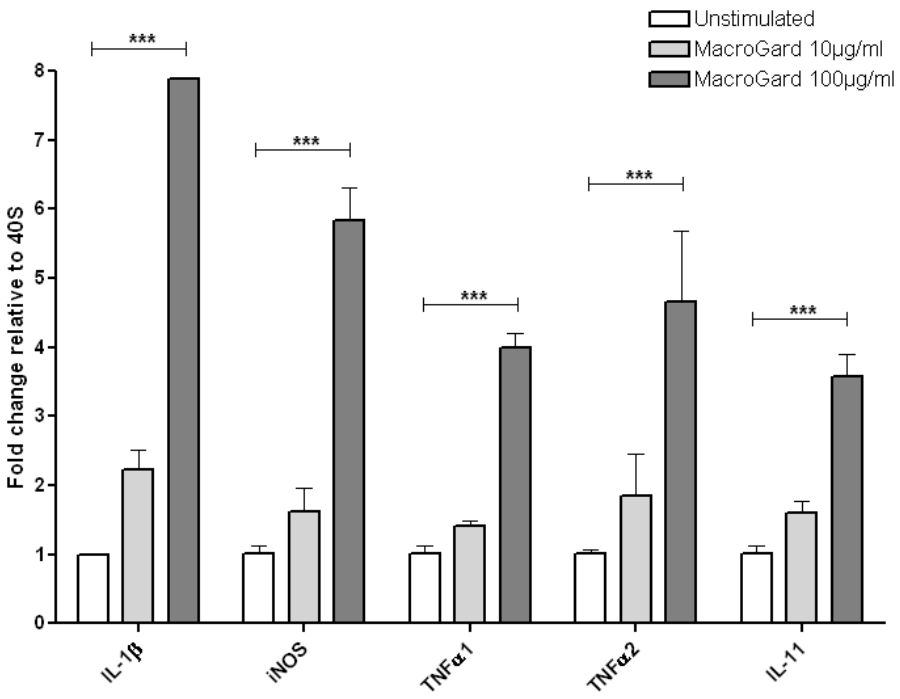


**Figure 10 Cytokine gene expression and iNOS gene expression from β-glucan stimulated carp Head kidney macrophages.** Gene expression was determined by real-time quantitative PCR. Carp HK derived macrophages were stimulated with 10 or 100 µg/ml MacroGard® during 6 hours. Results are expressed as fold change relative to 40S, data represents the mean value of two independent experiments and error bars show the standard error of the mean. Data sets were statistically compared by Man-Withney test, *** = P< 0.001. A list of the primers used is displayed in Appendix 1.

IL-1 has been reported to be the first signal that alerts surrounding cells to prevent further damage (Madhyastha et al., 2012). In mammalian models IL-1β induces the production of chemokines and growth factors by fibroblasts, keratinocytes and macrophages present in the wound (Hu et al., 2010a). Therefore it plays a major role in regulating inflammatory mediators production during wound healing processes (Hu et al., 2010b). *In vivo* studies in mechanically wounded rainbow trout measured an up-regulation of IL-1β at 4, 8 and 24 hours post injury (Ingerslev et al., 2010b). Furthermore, rainbow trout fibroblast cultures stimulated with DAMPs also showed up-regulation of IL-1β at 1, 4 and 24 hours post stimulation (Ingerslev et al., 2010b).Therefore, the IL-1β expression induced by β-glucan (figure 10) and tissue damage (table 2) might activate immune cells and resident cell types in the wound site. This activation could lead to the expression of other cytokines and growth factors, the production of reactive oxygen species and an enhanced leukocyte migration as reported in mammalian systems (Martin and Leibovich, 2005; Werner and Grose, 2003), stimulating wound healing in carp.

Tumor necrosis factor-α (TNF-α) is a major regulator of leukocyte migration and inflammation mainly produced by macrophages (Saeij et al., 2003). In carp two isoforms of TNF-α have been described (Saeij et al., 2003). The importance on the regulation of TNF-α during *Trypanoplasma borreli* infection in carp was demonstrated by the fact that depletion or over-expression of TNF-α led to higher mortality rates (Forlenza et al., 2009). The effect of TNF-α during fish wound healing has not been described. Son and co-workers demonstrated that β-glucan can activate murine macrophages to produce TNF-α, and suggest that TNF-α expression in the wound site would induce earlier inflammation and prompt pathogen removal from the wound. Furthermore, using an *in vitro* study, they showed that β-glucan can enhance murine fibroblast proliferation. Additionally, they proposed that faster fibroblast migration and TNF-α expression in the site of injury, could lead to improved wound healing (Son et al., 2007). During this study, β-glucan stimulation of CCB firoblasts did not enhance fibroblast proliferation nor migration (paper III), this could be explained by the difference of the β-glucan source [Water-soluble β-D-glucan extracted from *Aureobasidium* sp (Son et al., 2007), vs MacroGard® or zymosan (paper III)]. However, the production of TNF-α by β-glucan stimulated carp macrophages might enhance leukocyte infiltration to the wound site during the first phases of wound healing.

Interleukin-11 (IL-11) is a potent anti-inflammatory cytokine, involved in the proliferation and differentiation of hematopoietic progenitors, bone formation, adipogenesis, and protection of mucosal epithelia (Huising et al., 2005; Trepicchio et al., 1996).
In mammalian systems IL-11 has been shown to decrease mucosal damage and to accelerate wound healing by reducing macrophage cytokine and reactive oxygen species production (Huising et al., 2005; Mahboubi et al., 2000). IL-11 is produced by cells in an oxidative stress-dependent manner and has been linked to compensatory cell proliferation (Nishina et al., 2012). Additionally it has been suggested that IL-11 may exert its anti-inflammatory effects by reducing macrophage cytokine production and promoting immune deviation from Th1 to Th2 cytokine response (Mahboubi et al., 2000). In carp, the involvement of IL-11 in wound healing has not been described. However, PAMPs such as Lipopolysaccharide, Concavalin A and Peptidoglycan were shown to up-regulate IL-11 in carp HK macrophages (Huising et al., 2005; Ribeiro et al., 2010). β-glucan induces ROS (paper I and III) and NO in carp leukocytes (figure 9). Therefore, the observed IL-11 up-regulation upon stimulation of carp HK derived macrophages with β-glucan might be due to oxidative stress. But IL-11 could perhaps reduce cytokine and ROS production in the injury site and promote

compensatory cell proliferation as described in mammals. Still more research is needed to confirm this hypothesis.

# 4 Perspectives & future research

The work conducted during this thesis provides an insight into tissue repair and the use of β-glucan as immune modulator of wound healing in common carp. However, many further questions emerged from the outcome of the work presented here. Interesting studies can be performed in order to better understand such complex processes. These are among them.

## 4.4 More pieces for this puzzle

*In vitro* studies have shown to be very useful to investigate biochemical and physiological mechanisms (Hightower and Renfro, 1988; Lakra et al., 2011). During this thesis the role of fish fibroblasts and leukocytes was examined in relation to β-glucan stimulation and wound healing. Still, the synergistic effect of PAMPs and DAMPs, and other tissue cellular components such as malphigian cells and muscle fibres are interesting factors to include in future studies. Furthermore, the interaction between cellular components could be investigated trough the use of mixed-cultures, providing valuable information on the process of wound healing and immune modulation.

## 4.5 Let's go nano!

What if damage in a cell culture could be exerted at any specific place where the researcher would desire, and the ROS responses could be measured in real-time from the moment of injure? The use of cell culture nano-platforms as the one shown in figure 11, could allow the disruption of a single cell in a cell culture, and the monitoring of neighbouring cells responses. Furthermore, an intrinsic laminar flow in the platform could allow the stimulation of selected linear section with different molecules such as PAMPs or recombinant cytokines and to follow cell responses by specific cell retrieval for gene expression or proteomics analysis.

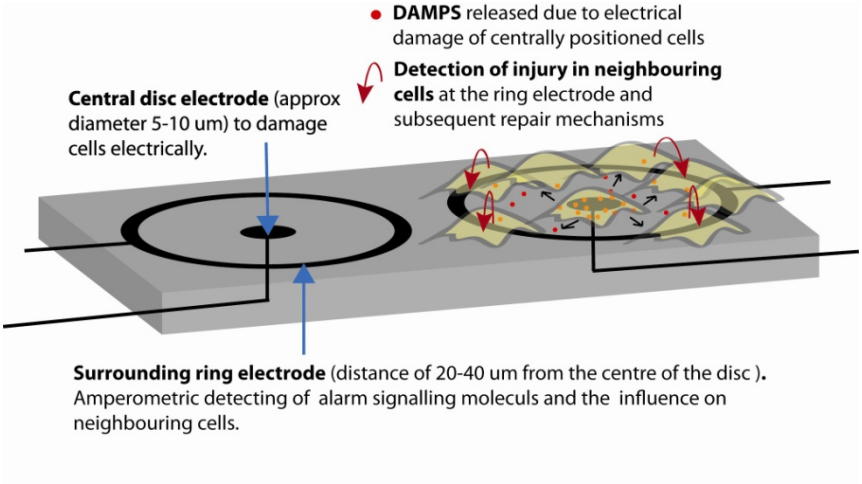


**Figure 11 Potential nano-platform design.** The would have the capability to electrically shock a single cell in a cell culture (situated on the central disc electrode), and subsequent electrochemical monitoring of the release of ROS and RNS from neighbouring cells (situated on the concentric ring electrodes)**. The design of this nano-platform and the presented figure is being carried out in close collaboration with Professor Jenny Emnéus form DTU Nanotech.**

### 4.6 Fibroblasts, more than a collagen factory

The importance of fish fibroblasts as producers of ECM products have been described (Zhang et al., 1998). Furthermore, Ingerslev and co-workers showed that rainbow trout fibroblasts express immune relevant genes after DAMP stimulation, contributing to inflammatory reaction during wound healing (Ingerslev et al., 2010b). Observations in mammalian cell systems have uncovered a fibroblast extra feature, they are able to phagocyte apoptotic neutrophils and collagen (Arlein et al., 1998; Bhide et al., 2005; Hall et al., 1994). Rainbow trout fibroblasts (RTHDF) failed to internalize latex beads during a phagocytic assay (Ingerslev et al., 2010b). However, during this study we could observe some fibrosis-like formations in wounded CCB fibroblasts, one day upon stimulation with MacroGard® (Fig.12). The fibrosis-like formations seem to enclose death fibroblasts (red stained in fig 12). Arlein and co-workers related collagen coated latex beads phagocytosis by murine fibroblast with cell elevated oxidation state (Arlein et al., 1998). So, could carp fibroblasts play a role also in clearance of death cells and/or foreign material?

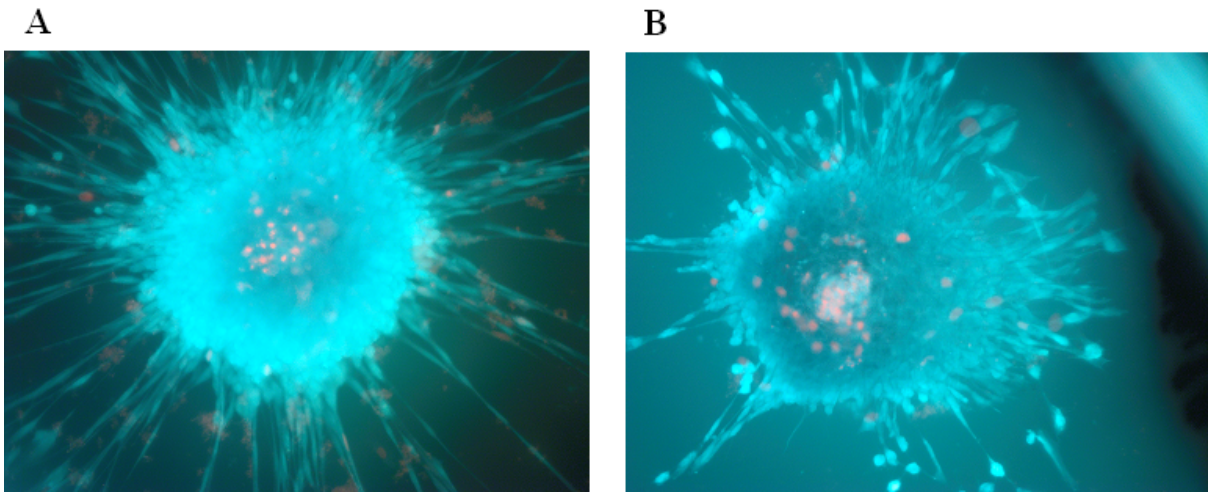


**Figure 12 Fibrosis-like formations in scratch-wounded CCB fibroblasts one day post-wounding.** CCB fibroblast monolayers were scratch wounded and stimulated with β-glucans,. One day posts wounding the cells were stained with fluorescein diacetate and propidium ioide. Green represents viable cells, red represents non-viable cells. Picture was made with the 20X Objective. **A)** Fibrosis-like formation in a 50 µg/ml MacroGard® stimulated scratch wound assay. **B)** Fibrosis-like formation in a 50 µg/ml MacroGard® stimulated scratch wound assay.

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# 6 List of Abbreviations

| | |
|---|---|
| Cat | Catalase |
| CCB | *Cyprinus carpio* brain |
| CR3 or CD11b/CD18 | Complement receptor type 3 |
| DAMP | Damage-Associated Molecular Pattern |
| ECM | Extracellular matrix |
| HK | Head kidney |
| HK-leukocytes | Head kidney leukocytes |
| HMBG1 | High-Mobility Group Box-1 |
| HSP | Heat Shock Protein |
| IFN- γ | Interferon- γ |
| IgM | Immunoglobulin M |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| Ip (injection) | Intraperitoneal injection |
| LacCer | Lactosylceramide |
| LPS | Liposaccharide |
| LTA | Lipoteichoic Acids |
| MQ-f | Macrophage-enriched fraction |
| Muc | Mucin |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NBT | Nitro-blue tetrazolium |
| NF-kB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NG-f | Neutrophilic granulocytes-enriched fraction |
| NK | Natural killer |
| NO | Nitric Oxide |
| PAMP | Pathogen-Associated Molecular Pattern |
| PDGF | Platelet-derived Growth Factor |
| PGN | Peptidoglycan |
| PRR | Pattern Recognition Receptor |
| ROS | Reactive Oxygen Species |
| RT-luminol | Real time luminol-enhanced chemiluminescence |
| SOD | Superoxide dismutase |
| SR | Scavenger Receptor |
| t-HK cells | Total head kidney cells |
| TGF-β | Transforming Growth Factor β |
| TLR | Toll-like Receptor |
| TNF- α | Tumor Necrosis Factor α |

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# 8 Appendix

**Primers used for Real-Time quantitative PCR presented in Section 3**

| Primer | Sequence (5′-3′) | GenBank Accession No. |
|---|---|---|
| **IL-1β Fw** | AAGGAGGCCAGTGGCTCTGT | AJ245635 |
| **IL-1β Rv** | CCTGAAGAAGAGGAGGAGGCTGTCA | |
| **IL-11 Fw** | CAGCAGCACAGCTCAGTACCA | AJ632159 |
| **IL-11 Rv** | AGCCTCTGCTCGGGTCATCT | |
| **iNOS Fw** | AACAGGTCTGAAAGGGAATCCA | AJ242906 |
| **iNOS Rv** | CATTATCTCTCATGTCCAGAGTCTCTTCT | |
| **TNF-α1 Fw** | GAGCTTCACGAGGACTAATAGACAGT | AJ311800 |
| **TNF-α1 Rv** | CTGCGGTAAGGGCAGCAATC | |
| **TNF-α2 Fw** | CGGCACGAGGAAGAAACCGAGC | AJ311801 |
| **TNF-α2 Rv** | CATCGTTGTGTCTGTTAGTAAGTTC | |
| **40S Fw** | CCGTGGGTGACATCGTTACA | AB012087 |
| **40S Rv** | TCAGGACATTGAACCTCACTGTCT | |

# 9 Accompanying papers

**PAPER I**

# Comparative study of ß-glucan induced respiratory burst measured by Nitroblue tetrazolium assay and Real-time luminol-enhanced chemiluminescence assay in common carp (*Cyprinus carpio* L.)

**N. I. Vera-Jiménez [1], D. Pietretti[2], G.F. Wiegertjes[2] and M.E. Nielsen[1]**

**Address correspondence and reprint requests to** Nielsen ME. DTU Food, National Food Institute, Biological Quality Research Group, Søltofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark. Telephone number: +45-45254906. E-mail address: mice@food.dtu.dk

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Comparative study of β-glucan induced respiratory burst measured by nitroblue tetrazolium assay and real-time luminol-enhanced chemiluminescence assay in common carp (*Cyprinus carpio* L.)

N.I. Vera-Jimenez [a], D. Pietretti [b], G.F. Wiegertjes [b], M.E. Nielsen [a,*]

[a] *DTU Food, National Food Institute, Division for Industrial Food Technology, Biological Quality Research Group, Søltofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark*
[b] *Cell Biology and Immunology Group, Wageningen Institute of Animal Sciences, Wageningen University, PO Box 338, 6700 AH Wageningen, The Netherlands*

**Abstract**

The respiratory burst is an important feature of the immune system. The increase in cellular oxygen uptake that marks the initiation of the respiratory burst is followed by the production of reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide which play a role in the clearance of pathogens and tissue regeneration processes. Therefore, the respiratory burst and associated ROS constitute important indicators of fish health status. This paper compares two methods for quantitation of ROS produced during the respiratory burst in common carp: the widely used, single-point measurement based on the intracellular reduction of nitroblue tetrazolium (NBT) and a real-time luminol-enhanced assay based on the detection of native chemiluminescence. Both assays allowed for detection of dose-dependent changes in magnitude of the respiratory burst response induced by β-glucans in head kidney cells of carp. However, whereas the NBT assay was shown to detect the production of only superoxide anions, the real-time luminol-enhanced assay could detect the production of both superoxide anions and hydrogen peroxide. Only the chemiluminescence assay could reliably record the production of ROS on a real-time scale at frequent and continual time intervals for time course experiments, providing more detailed information on the respiratory burst response. The real-time chemiluminescence assay was used to measure respiratory burst activity in macrophage and neutrophilic granulocyte-enriched head kidney cell fractions and total head kidney cell suspensions and proved to be a fast, reliable, automated multiwell microplate assay to quantitate fish health status modulated by β-glucans.

**Keywords:** *Respiratory burst kinetics; NBT assay; Real-time luminol assay; Common carp; Superoxide anion; Hydrogen peroxide; β-glucans.*

## 1. Introduction

Multicellular organisms mediate their early defence against pathogens based on their innate immune system, which provides them with the ability to recognize the presence of pathogens and react rapidly against them [1, 2]. The common carp, *Cyprinus carpio*, has been intensive studied for many purposes. Common carp is worldwide the most cultured fish species for food consumption. It represents one of the most important species used in aquaculture and although many studies have focused on physiological aspects such as nutrition, farming conditions and infectious diseases [3-6], it is important to develop and improve reliable methods to monitor and control the health status of carp. The respiratory burst is regarded as one of the most important early defence mechanisms as it plays a crucial role in pathogen eradication, but has also been shown to be involved in tissue regeneration. Therefore, the respiratory burst is a significant mechanism that can be used to monitor health status in fish [7-9].

Several studies have dealt with the ability of phagocytes to recognize pathogens through the detection of pathogen-associated molecular patterns (PAMPs), which are highly conserved molecules not generally expressed in higher organisms [10, 11]. Phagocytes have also been related to the recognition of damage-associated molecular patterns (DAMPs), those being self signals of tissue damage and cell death [10, 12]. The recognition of all these molecules occurs using special receptors called pattern-recognition receptors (PRRs), and trigger a series of events including the respiratory burst [10, 13-15]. The initiation of the respiratory burst is marked by an increase in oxygen cellular uptake, followed by the one electron reduction of molecular oxygen ($O_2$) to superoxide anions ($O_2^-$). This reaction is catalysed by the membrane-associated enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, using NADPH as the electron donor [9, 16-19]. Further reduction of oxygen produces hydrogen peroxide ($H_2O_2$), which occurs either as a spontaneous dismutation, especially at low pH, or as a catalyzed reaction by a family of enzymes called Superoxide dismutase (SOD). Additional reactions of $O_2^-$ and $H_2O_2$ may lead to the formation of hydroxyl radicals ($OH^-$), especially in the presence of iron through the Fenton or Haber-Weiss reactions. The interaction of $H_2O_2$ with myeloperoxidase (MPO) can produce hypochlorous acid and other toxic metabolites if $H_2O_2$ is not dismutated to water and molecular oxygen by the enzyme catalase that can act as a natural scavenger [16, 20-24] (See Fig.1). Although different techniques for the quantiation of the respiratory burst have been developed through the years, comparisons of the accuracy and reliability to evaluate fish health status among those techniques are scarce.

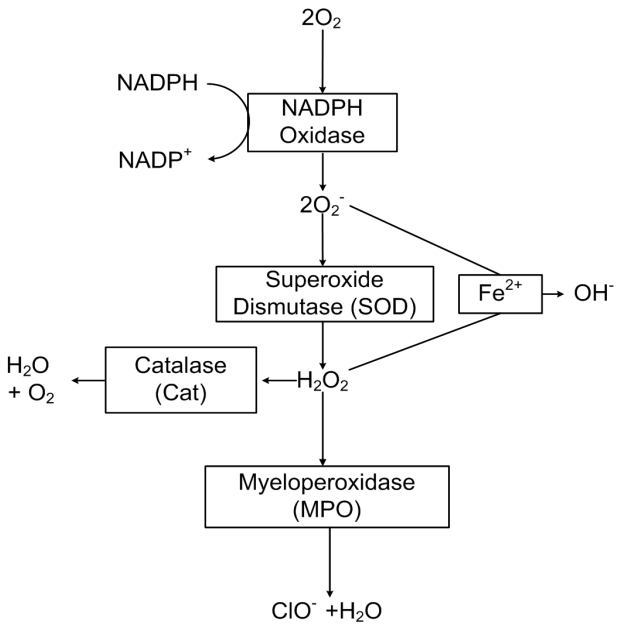



**Figure 1. Schematic representation of the Respiratory burst main products**. The membrane-associated enzyme nicotinamide adenine dinucleotide phosphate (NADPH) catalyzes the reduction of molecular oxygen ($O_2$) to superoxide anion ($O_2^-$), using NADPH as the electron donor. Further reduction of oxygen produces hydrogen peroxide ($H_2O_2$), which occurs either as a spontaneous dismutation, or as a catalyzed reaction by a family of enzymes called Superoxide dismutase (SOD). Additional reactions of $O_2^-$ and $H_2O_2$ may lead to the formation of hydroxyl radicals ($OH^-$), especially in the presence of iron through the Fenton or Haber-Weiss reactions. The interaction of $H_2O_2$ with the enzyme myeloperoxidase (MPO) can produce hypochlorous acid and other toxic metabolites, or $H_2O_2$ is dismutated to water and molecular oxygen by the enzyme catalase.

To date, several methodologies for the measurement of respiratory burst have been described. Initially, Babior et al. (1973) assessed extracellular O2- based on its capability to reduce ferricytochrome c, reading absorbance at 550 nm. The main disadvantage of this methodology was its limitation to follow the kinetics of the reaction. This restrain was overcome by Cohen and Chovaniec (1978) by introducing the continuous recording of absorbance in a cell suspension, however both methods require large amounts of cells (≈2.5x106 cells/well) and reagents (≈950 µl/well) [19, 25-27]. In parallel, Root et al. (1975) formulated a new methodology for the calculation of respiratory burst produced by human granulocytes; in this procedure the loss of fluorescence of scopoletin (7-OH-6-methoxycoumarin), a natural compound found in the root of plants in the genus *Scopolia*), was evaluated after exposure of $H_2O_2$ in the presence of horseradish peroxidase (HRP). This technique provided high detection sensitivity (as little as 0.2 nmoles $H_2O_2$/ml), but real-time measurements remained problematic due to the rapid diminution of scopoletin concentration in the samples. Furthermore, the technique cannot easily be applied to adherent cells, since it required the establishment of the cultures in flying coverslips which are then placed in the spectrofluorometer cuvette in a certain standard position [28, 29]. Pick and Keisari (1980) and Pick and Mizel (1981) established two detection methods based on the HRP-dependent oxidation of phenol sulfonephthalein (phenol red), and a combination of the phenol red and cytochrome c assay, respectively. These methodologies allowed them to measure respiratory burst in macrophage cultures of guinea pigs. However, the sensitivity of the $H_2O_2$ detection was reduced to 1 nmoles/ml [19, 29]. The most successful alternative was developed by Baehner and Nathan (1968) who introduced the use of nitroblue tetrazolium (NBT) in the detection of respiratory burst [30]. The NBT assay protocol has been optimized over the years but its principle has remained the same [17, 31-37]. NBT is a yellow, water soluble substance which is internalized by phagocytes, and then reduced intracellularly to formazan

during the respiratory burst. For quantitation, the cell membrane is disrupted, the formazan is dissolved in KOH and the absorbance is read from 509 to 690 nm [30, 31, 33, 34, 37]. NBT has perhaps become the most popular method for monitoring respiratory burst to various stimuli [38]. However, inconveniences associated with the NBT assay such as the impossibility to measure real-time during the respiratory burst process and its laborious protocol which increases the risk of pipetting errors, therefore decreasing accuracy, have remained an issue.

Allen et al. described a different approach for the detection of respiratory burst for human polymorphonuclear leukocytes already in 1972. In this study, the authors describe the occurrence of electronically excited states during the production and transformation of free radicals in the respiratory burst. Furthermore, they observed that after electron relaxation to their initial ground state, energy was release in form of photons. This process is known now as native chemiluminescence and can be amplified for its detection using luminol [39, 40]. Different protocols for the luminol amplification of radical production have been used through the years in different species [41-44]. This paper, for the first time, compares the popular NBT method with the native chemiluminescence amplification method for use in carp (*Cyprinus carpio*). Using β-glucans to induce a respiratory burst response in head kidney leukocytes, the accuracy, sensitivity and adaptability of both methodologies are examined and compared, and their use to quantitate fish health status is discussed.

## 2. Materials and Methods

### *2.1 Fish*

European Common carp (*Cyprinus carpio carpio*) were obtained from the central fish facility 'De Haar-vissen' (Wageningen, The Netherlands). R3xR8 carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and the Polish origin (R3) [45]. The fish used ranged between 50 to 100 g and were kept at 23°C (±1°C) with 12:12 h light: dark photoperiod.

### *2.2 Preparation of head kidney cell suspensions:*

During this study four different head kidney cell suspensions were used, they are referred to throughout the paper as: total head kidney cell suspension (t-HK cells), head kidney leukocyte suspension (HK-Leukocyte), macrophage-enriched fraction cell suspension (MQ-f) and neutrophilic granulocyte-enriched fraction cell suspension (NG-f). To isolate the cells carps were euthanized using an overdose of MS-222 (100 mg/l). Fish were bled from the caudal vein, both head-kidneys were excised and placed in a 100 µm nylon cell strainer (BD Falcon). The purification processes used to obtain the different cell suspensions are explained below.

Total head-kidney cell suspensions (t-HK cells) were obtained by pressing the head-kidney with a plunger through the cell strainer, the cells collected were rinsed with phenol red-free Hank's balanced salt solution (HBSS, Sigma-Aldrich, Cat nr. H8264).

Head kidney leukocyte (HK-Leukocyte) suspensions were obtained using a non-continuous percoll (Sigma-Aldrich, Cat nr. P4937) gradient based on the protocol described by Kemenade et al. [46]. Briefly, percoll layers of 1,02 and 1,08 g/ml were used. After 25 minutes centrifugation at 800*g*, the cells present in the 1,02-1,08 interface were collected, washed three times and resuspended with HBSS. Cell viability was assessed by Trypan Blue exclusion (Sigma-Aldrich, Cat nr. T8154).

Macrophage and granulocyte enriched fractions, were obtained using non-continuous percoll gradient with percoll layer concentrations of 1.2; 1.06; 1.07 and 1.083 g/ml. Cells present in the 1.06-1.07 and the 1.07-1.083 interfaces were collected, representing the macrophage-enriched fraction (MQ-f) and the neutrophilic granulocyte-enriched fraction (NG-f) respectively [46]. As explained previously, the cell fractions were washed, resuspended in HBSS and cell viability was assessed.

*2.3 Preparation of β-glucans*

During this study two different β-glucans were used: MacroGard®, which is a bakers' yeast extract containing a 60% purified fraction of 1,3/1,6 β-glucan [47], and Zymosan a glucan molecule with repeating glucose units connected by β-1,3 glycosidic linkages. Both β-glucans have shown to trigger respiratory burst in different cell populations and several fish species [48, 49].

Stock solutions of MacroGard® (Biorigin) and Zymosan A (Sigma-Aldrich, Cat nr. Z4250) were prepared in milliQ water (PURELAB Ultra, Elga) at 20 mg/ml and 10 mg/ml respectively and sonicated twice during 30 seconds using power 6 of a Brandson sonifier 250. Subsequently, the sonicated solutions were pasteurized using a thermoblock at 80°C during 20 minutes.

*2.4 Single point measurement of reactive oxygen species: Nitroblue Tetrazolium (NBT) assay*

The NBT analysis was performed as previously described [36]. Briefly, cells were brought to $10X10^6$ cells/ml in RPMI medium (Sigma-Aldrich, Cat nr. R7509). Cell monolayers were prepared in a 96-well tissue culture plate (Corning®, Cat nr. 3300) by applying 100µl/well of the cell suspension. Cells were incubated during 1 hour at 26°C with 5% $CO_2$, after the incubation time, cells were washed twice with phenol red-free Hank's balanced salt solution (HBSS) and the respiratory burst was induced and measured. In general, 160µl of RPMI containing NBT (1 mg/ml. Sigma-Aldrich, Cat nr. N6876) were added to each well in a plate seeded with t-HK cells. To induce respiratory burst 10µl of stimulus (either MacroGard® or Zymosan) were used. Plates were incubated at 26°C with 5% $CO_2$ during 60 minutes. After

the incubation time was completed, the plates were washed once with 100 µl of RPMI medium and cells were fixed adding 100% methanol during 3 minutes. Subsequently, two washes in 70% ethanol were made and plates were allowed to air-dry. The reduced formazan was dissolved in 120 µl KOH (2M), and cells were lysed adding 140 µl dimethyl sulphoxide (DMSO Sigma-Aldrich, Cat nr. D2650). The reduction of NBT in each well was then measured at 690 nm with the reference filter 414nm using a multimode microplate reader (Synergy 2, Biotek).

To identify the type of reactive oxygen species measured by the NBT assay, total head kidney cells were first stimulated with 100 µg/ml MacroGard® and then treated with either catalase (Cat, 300 U/ml, Sigma-Aldrich, Cat nr. C1345) to provoke the dismutation of hydrogen peroxide to water and oxygen [24]or with superoxide dismutase (SOD, 250 U/ml, Sigma-Aldrich, Cat nr. S5395) to catalyze the dismutation of superoxide to hydrogen peroxide and oxygen [27].

To monitor the sensitivity and adaptability of the NBT assay to quantitate effects of different doses of β-glucans and different cell suspensions, monolayers of t-HK cells or HK-Leukocytes were seeded in 96-well plates. Cells were stimulated with 10, 50 or 100 µg/ml of MacroGard® or Zymosan and incubated during 30, 45, 90 or 180 minutes at 26°C with 5% $CO_2$. After the incubation time was completed the dissolution of formazan crystals was measured.

*2.4 Real-time luminol-enhanced chemiluminescence assay (RT-luminol assay):*

The RT-luminol assay is based on a protocol described by Allen *et al.* and later modified by Verho *et al*.; this method amplifies the native chemiluminescence produced during the respiratory burst using luminol [39, 50]. In general, white 96-well plates (Corning®, Cat nr. 3917) were prepared containing 40 µl of luminol (10mM, Sigma-Aldrich, Cat nr. A8511) in 0.2 M borate buffer (pH 9.0) and 100 µl of stimulus for the induction of respiratory burst (either MacroGard® or Zymosan), subsequently the volume of the wells was adjusted to 200 µl using HBSS. Head kidney cell suspensions were added at a concentration of $0.5X10^6$ cells/well in all the experiments performed, the final volume of each well was always 300 µl. The chemiluminescence emission of the cells was measured with a luminometer (synergy2, Biotek) every 3 minutes at 26°C.

To identify the type of reactive oxygen species measured by the RT-luminol assay, total head kidney cells were first stimulated with 100 µg/ml MacroGard® and then treated with either catalase (Cat, 300 U/ml), to provoke the dismutation of hydrogen peroxide to water and oxygen, [24] or with superoxide dismutase (SOD, 250 U/ml) to catalyze the dismutation of superoxide to hydrogen peroxide and oxygen [27]. The chemiluminescence emission of the cells is expressed as the integral of the relative light units (Max RLU) recorded by the luminometer between 0 and 60 minutes.

To monitor dose-dependent sensitivity of the RT-luminol assay and the adaptability of this method to different cell suspensions, t-HK cells and HK-Leukocytes were stimulated with MacroGard® or Zymosan (10, 50 or 100 µg/ml). The chemiluminescence emissions are expressed as relative light units (RLU) recorded during 210 minutes.

To quantitate ROS production by different phagocyte sub-populations, macrophage-enriched fractions (MQ-f) and neutrophilic granulocyte-enriched fractions (NG-f) from carp head kidney, were stimulated with MacroGard® or Zymosan (100 µg/ml). Results are expressed as RLU recorded during 210 minutes.

*2.5 Statistical analysis*

The software GraphPad Prism (version 4.03) was used for statistical work. Statistical comparison was performed by one-way analysis of variance (ANOVA) and further Bonferroni post-tests. In experiments involving ROS kinetics a two-way ANOVA analysis and Bonferroni post-tests were used (tables with significant differences are included as supplementary data). $P<0.05$ was considered to be statistically significant.

## 3. Results

*3.1 The RT-luminol assay detects reactive oxygen species additional to those detected by the NBT assay*

The NBT assay after stimulation of t-HK cells with MacroGard®, showed an increase of oxygen radical production. Cells treated with catalase did not indicate major changes to the production of oxygen radicals. Conversely, treatment with SOD, showed a markedly reduced magnitude of ROS. As expected, co-treatment with SOD and catalase also decreased ROS production (see figure 2A).

The RT-luminol assay showed an increase in the oxygen radicals produced by t-HK cells after stimulation with MacroGard®. Cell treatment with SOD or the combination of SOD and catalase markedly decrease ROS production. In addition, treatment with catalase also showed a significant reduction of oxygen radical production (see figure 2B).

In conclusion, the NBT assay was shown to detect the production of only superoxide anions, the RT-luminol assay could detect the production of superoxide anions, hydrogen peroxide and related radicals.

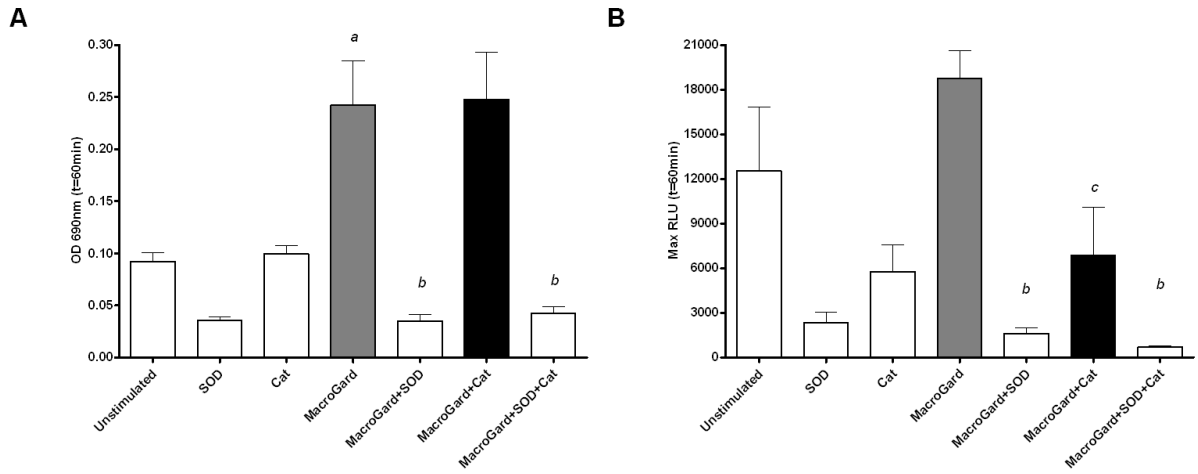


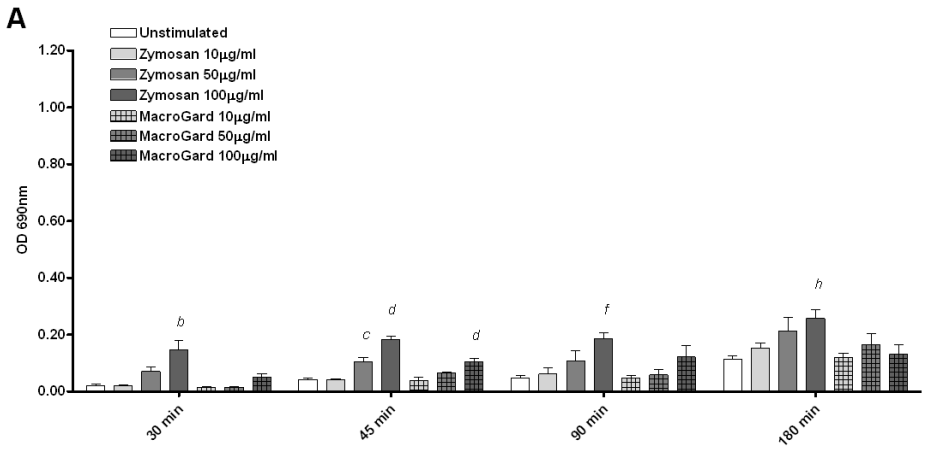

**Figure 2**. **Identification of the oxygen radicals measured by NBT and RT-luminol assay.** Comparison of the nitro blue tetrazolium NBT assay (A) and the Real-time luminol assay (B) in relation to the type of oxygen radicals being produce after stimulation of t-HK cells with MacroGard® (100µg/ml) and co-stimulation with SOD (250 U/ml) or catalase (300U/ml). The plots show the mean value of four independent studies for NBT and five for RT-luminol assays. Error bars represent standard error of the mean. Statistical comparison was performed by one-way ANOVA. *a*= significant difference to unstimulated sample with P< 0.01, *b*= significant difference to MacroGard® with P< 0.001 and *c*= significant difference to MacroGard® with P< 0.05.

### *3.2 Dose-effect sensitivity and adaptability of the methods.*

The adaptability of the NBT and RT-luminol assays to different cell groups, and their sensitivity to dose-related changes in the respiratory burst response, were examined following stimulation of t-HK cells and HK-Leukocytes with different β-glucans doses (10, 50 and 100 µg/ml).

Measurements of the respiratory burst response of t-HK and HK-Leukocytes by NBT are plotted in figure 3A and 3B respectively. Following stimulation with β-glucans, a higher oxygen radical production was elicited in HK-Leukocytes than in t-HK cells. Variations in the magnitude of oxygen radical production related to changes of the β-glucan doses were detectable using NBT in both cell groups.

The RT-luminol assay measurements of the respiratory burst response after β-glucan stimulation of t-HK cells and HK-Leukocytes are plotted in figure 3C and figure 3D respectively. The oxygen radical production elicited by β-glucans in t-HK cells, showed to be higher than the one from HK-Leukocytes. Differences in oxygen radical production due to the β-glucan doses were clearly identified in both cell groups. Furthermore, since the measurements of this method are made continuously, a peak of oxygen radical production was determined 36 minutes post-stimulation for t-HK cells, and 57 minutes post-stimulation for HK-Leukocytes. .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Zymosan 50µg/ml
Zymosan 100µg/ml
MacroGard 10µg/ml
MacroGard 50µg/ml
MacroGard 100µg/ml
OD 690nm
1.20
1.00
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0.60
0.40
0.20
0.00
b
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d
d
f
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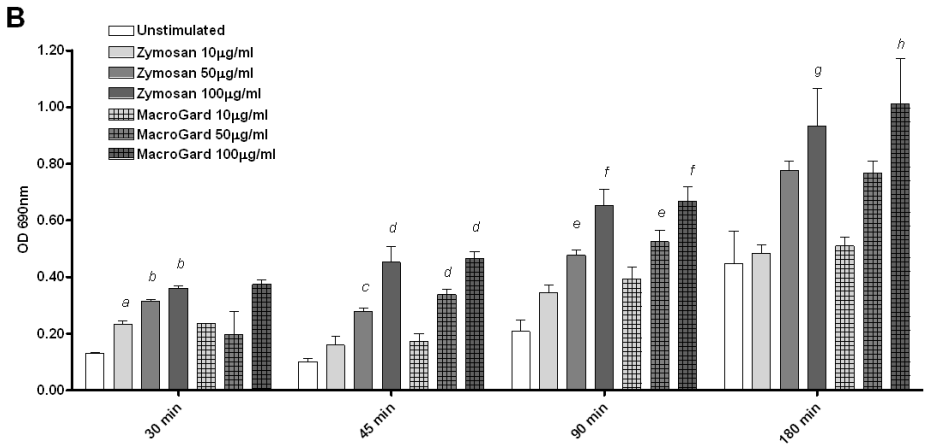
30 min
45 min
90 min
180 min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MacroGard 10µg/ml
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0.80
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0.40
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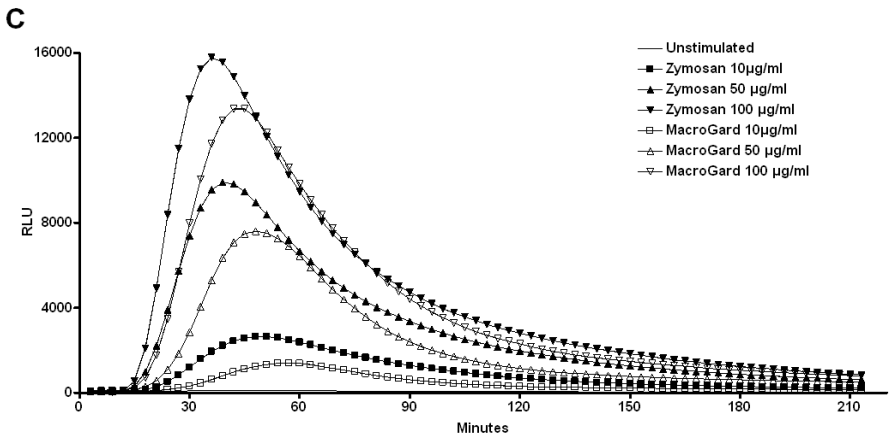
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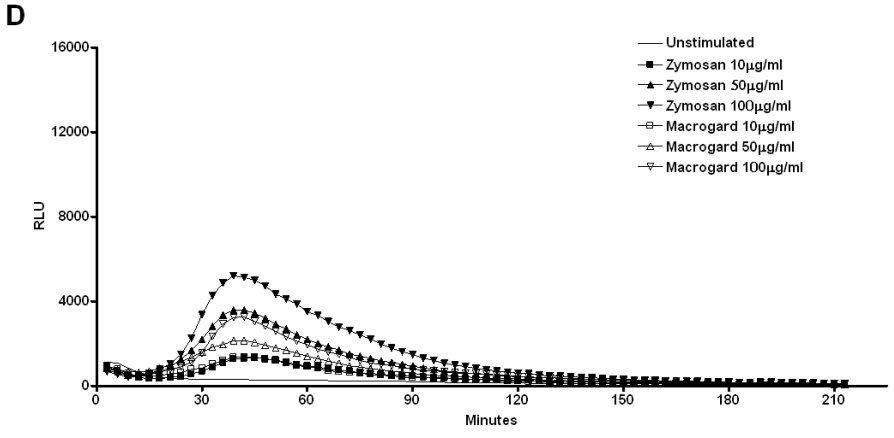
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Macrogard 10µg/ml
Macrogard 50µg/ml
Macrogard 100µg/ml
RLU
16000
12000
8000
4000
0
0
30
60
90
120
150
180
210
Minutes

**Figure 3. Comparison of the respiratory burst activity measured using NBT and RT-luminol assay.** NBT results of (A) t-HK cells and (B) HK-Leukocytes after stimulation with 10, 50 or 100 µg/ml of Zymosan or MacroGard®. NBT was measured at 30, 45, 90 and 180 minutes. The plots show the mean value of four independent studies for all the NBT time points, except for HK-Leukocytes 30 minutes where two independent studies are plotted. Statistical comparison was performed by one-way ANOVA. *a*= significant difference to 30 min unstimulated with P< 0.01, *b*= significant difference to 30 min unstimulated with P< 0.001, *c*= significant difference to 45 min unstimulated with P< 0.05, *d*= significant difference to 45 min unstimulated with P< 0.001, *e*= significant difference to 90 min unstimulated with P< 0.01, *f*= significant difference to 90 min unstimulated with P< 0.001 *g*= significant difference to 180 min unstimulated with P< 0.01, *h*= significant difference to 180 min unstimulated with P< 0.05.Continuous measurements of oxygen radical production by RT-luminol assay are shown in (C) for t-HK cells and (D) for HK-Leukocytes after stimulation with 10, 50 or 100 µg/ml of Zymosan or MacroGard®, chemiluminescence was monitored every three minutes during 210 minutes. The plots show the mean value of four independent RT-luminol assays in t-HK cells and 3 in HK-Leukocytes. Statistical comparison was performed by two-way ANOVA, tables showing significant differences are inlcuded as supplementary data.

Summarizing, both methodologies studied showed the capacity to measure respiratory burst following stimulation with β-glucan. NBT and RT-luminol assay were responsive to changes of the β-glucan doses and correlated in the magnitude of the response. However, only RT-luminol offered measurements at frequent and continual time intervals during the course of the experiments, providing information on the stimulation peaks and the respiratory burst kinetics

### *3.3 RT-luminol assay measurements of respiratory burst response in macrophage enriched and neutrophilic granulocyte -enriched fractions after β-glucan stimulation.*

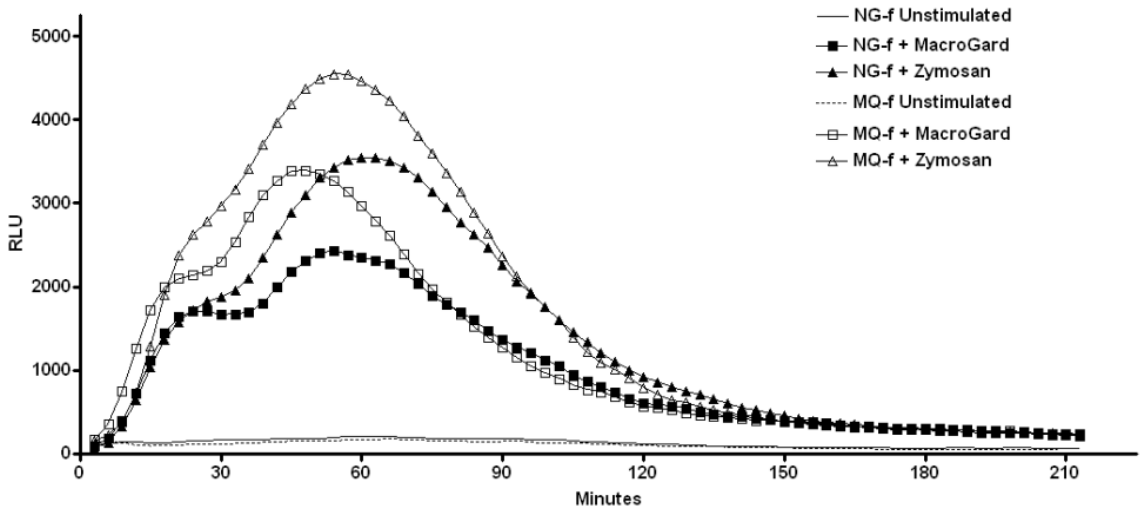


**Figure 4. RT-luminol assay on further purified head kidney fractions.** Comparison of the respiratory burst measurements by RT-luminol assay in two different cell fractions of carp head kidney cells. Macrophage-enriched fraction (MQ-f) and neutrophil granulocyte-enriched fraction (NG-f) were stimulated with either MacroGard® 100 µg/ml, Zymosan 100 µg/ml or left untreated. Luminescence was continuously monitored for 210 minutes. The graph shows the mean value of two independent studies. Statistical comparison was performed by two-way ANOVA, tables showing significant differences are inlcuded as supplementary data.

To evaluate the adaptability of the RT-luminol assay to further purified head-kidney phagocyte sub-populations, the respiratory burst response of MQ-f and NG-f was measured after stimulation with β-glucans.

The respiratory burst response of MQ-f and NG-f to β-glucans was detectable and is plotted in figure 4. Higher production of oxygen radicals was recorded from MQ-f than from NG-f after stimulation with β-glucans. The results also displayed a minor wave present between 15 and 35 minutes for both cell fractions.

## 4 Discussion

The present study compares the use of NBT and RT-luminol assays for the assessment of oxygen radical production in carp after stimulation with β-glucans, a PAMP known to stimulate the respiratory burst in fish and mammalian systems [48, 51]. Both methods were able to detect the production of oxygen radicals after stimulation with MacroGard® and Zymosan, and allowed the detection of dose-dependent changes on the respiratory burst magnitude. On this basis, both methods can be used not only to study the respiratory burst responses during microbicidal events, but also to study the effect of immune-stimulants, vaccines and pharmacological agents on the immune-system [50, 52, 53]. However, one of the major differences between the methods compared in this study consisted on the possibility to follow the kinetics of the respiratory burst response. Since the NBT assay is based on the intracellular reduction of the nitroblue tetrazolium salt by the superoxide anion ($O_2^-$) [31], the cells had to be lysed to perform the measurements. It implies that only a one time-point measurement can be retrieved by this method, expressed by the accumulative value of oxygen radicals produced intracellularly during a set period of time. On the other hand, the RT luminol assay amplifies the native chemiluminescence produced during the respiratory burst process at any given instant. This allows the tracking of the reaction kinetics, and makes possible the identification of oxygen radical production peaks. Furthermore, due to its chemical structure luminol can cross biological membranes, allowing the detection of extracellular and intracellular production of oxygen radicals [54]. During this study several time-points were measured using NBT to produce a kinetic profile of the respiratory burst. However, due to the accumulative nature of its data, peaks of oxygen radical production could not be identified. Furthermore, the amount of cells required for the experiment was at least 4 times higher than the one used with the RT-luminol assay. The lab-work involved in the NBT measurements is considerable more time consuming than the one for RT-luminol. Sample handling during NBT development is more extensive, therefore the risk of pipetting mistakes increases.

The reduction in the magnitude of the respiratory burst response after addition of SOD, a scavenger of superoxide anion ($O_2^-$) [27], showed that NBT and RT-luminol assay detect $O_2^-$.

The use of catalase, an enzyme which catalyses the dismutation of hydrogen peroxide to water and oxygen [24], evidenced the incapability of NBT to detect other radicals than $O_2^-$. Therefore, it suggests the NBT as a semi quantitative method. On the contrary, the RT-luminol assay successfully detected the variation on the respiratory burst from carp head-kidney cells after the addition of catalase. Thus, RT-luminol can detect hydrogen peroxide and related radicals such as hypochlorous acid and hydroxyl radical. The type of radicals being measured by this two methods had not been compared using fish cells. However, the results of this study are in agreement with Cheson *et al*., [55] who postulated that, the light emitted by human phagocytosing granulocytes came from the oxidative capacity of any of the oxidizing agents released by the cells. Furthermore, Schopf *et al.* [37] attributed the luminescence of human monocytes and polymorphonuclear leukocytes directly to the oxidizing properties of superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals.

Both, the NBT and the RT-luminol assays measured the respiratory burst response triggered by β-glucans independently of the cell suspension used. It indicates the adaptability of the methods to different degrees of cell purification. However, higher values of respiratory burst response of HK-Leukocytes were measured by NBT when compared to t-HK cells. Such difference in the magnitude of the response might be related to the activation of the leukocytes due to the purification process. Interestingly the RT-luminol assay evidenced higher production of oxygen radicals of t-HK cells when compared to HK-Leukocytes. This augment in the respiratory burst response of t-HK cells might be explained by the presence of damage associated molecular patterns (DAMPs). Different studies have examined the ability of DAMPs to trigger immune responses [40] and their synergism with pathogen-associated molecular patterns (PAMPs) [56]. Furthermore, non-immune cells such as epithelial cells, fibroblasts and erythrocytes have been shown to produce reactive oxygen species [57-59], and to interact with different cell types to enhance the magnitude of the respiratory burst response [60, 61]. Since the t-HK cell suspensions used in this study did not have any purification process, it contained healthy head kidney cells (not only leukocytes) mixed with necrotic, damaged cells, and debris from the tissue. The synergistic effect of PAMPs and DAMPs, added to the collaboration of different cell types in the cell suspension, could cause the higher respiratory burst response evidenced with the RT-luminol assay. The fact that t-HK cell suspensions did not show an increase of the respiratory burst response when measured by NBT could be explained by the limitation of this method to detect hydrogen peroxide. Cell proliferation and tissue regeneration processes have been linked to the presence of $H_2O_2$ in mammalian models [62, 63]. In addition, a gradient of hydrogen peroxide has been reported in zebra fish after tissue injure [8]. Therefore, it would be logical to think that the DAMPs present in the cell suspensions, could trigger the production of messengers for tissue regeneration such as hydrogen peroxide, which was detected by RT-luminol assay but not by NBT.

Finally, the kinetics feature of the RT-luminol allowed the identification of a minor biphasic response during the oxygen radical production of β-glucan stimulated MQ-f and NG-f. This response could be associated to the adhesion and ingestion phases of the phagocytosis. Nikoskelainen *et.al* [64] described these two phases during the respiratory burst induced by *Aeromonas salmonicida* in rainbow trout phagocytes. The authors claimed that those phases cannot be distinguished in rainbow trout when the number of head kidney cells exceeds $5x10^4$ cells/well because the peaks are merged [64]. During this study, the number of carp MQ-f and NG-f was higher ($5x10^5$ cells/well). However, the fact that a similar response was observed may be due to the difference in fish species, as discussed by various authors for other immune responses [1, 65].

## 5 Conclusions

Both of the methods compared during this study, showed the capacity to detect and measure the respiratory burst response of carp head kidney cells after stimulation with β-glucans, therefore constitute an indicator of the general fish health status. However, only the RT-luminol assay allowed the tracking of kinetics during the respiratory burst response, offering information about peaks of oxygen radical production. Furthermore, only the RT-luminol assay detected the production of hydrogen peroxide and oxygen related radicals, becoming an important tool to monitor production of oxygen radicals involved in tissue regeneration processes. The RT-luminol assay also proved to be a simple and fast protocol which reduces sample manipulation, requires fewer amounts of cells per experiment, and can be used to evaluate the respiratory burst responses from mixed cell populations to highly purified subpopulations.

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## Supplementary data

**Table 1. Statistical significance of the t-HK respiratory burst activity measured by RT-luminol.** Table shows the significant differences among treatments after two-way ANOVA analysis and Bonferroni post-tests. * = P< 0.05, ** = P< 0.01, *** = P< 0.001, ns= no significant difference.

| | Minutes | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | 0-15 | 18 | 21 | 24 | 27 | 30-33 | 36 | 39 | 42-60 | 63-69 | 72 | 75-90 | 93-96 | 99 | 102-111 | 114-120 | 123-126 | 129-135 | 138-144 | 147-153 | 156-213 |
| Zymosan 10µg/ml | ns | ns | ns | ns | ns | ns | * | ** | *** | ** | * | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Zymosan 50µg/ml | ns | ns | ** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | ** | * | ns | ns | ns | ns |
| Zymosan 100µg/ml | ns | ** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | ** | * | ns |
| MacroGard 10µg/ml | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| MacroGard 50µg/ml | ns | ns | ns | ns | * | *** | *** | *** | *** | *** | *** | *** | ** | * | ns | ns | ns | ns | ns | ns | ns |
| MacroGard 100µg/ml | ns | ns | * | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | ** | * | ns | ns | ns |

**Table 2. Statistical significance of the HK-Leukocytes respiratory burst activity measured by RT-luminol.** Table shows the significant differences among treatments after two-way ANOVA analysis and Bonferroni post-tests. * = P< 0.05, ** = P< 0.01, *** = P< 0.001, ns= no significant difference.

| | Minutes | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | 0-24 | 27 | 30 | 33 | 36 | 39-45 | 48-51 | 54 | 57 | 60 | 63 | 66-75 | 78 | 81 | 84-213 |
| Zymosan 10µg/ml | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Zymosan 50µg/ml | ns | ns | ** | *** | *** | *** | *** | *** | *** | ** | * | ns | ns | ns | ns |
| Zymosan 100µg/ml | ns | ** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | ** | * | ns |
| MacroGard 10µg/ml | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| MacroGard 50µg/ml | ns | ns | ns | ns | ns | * | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| MacroGard 100µg/ml | ns | ns | ns | ** | *** | *** | *** | ** | * | * | ns | ns | ns | ns | ns |

**Table 3. Statistical significance of the neutrophil granulocyte (NG)-enriched fraction and macrophage (MQ)-enriched fraction respiratory burst activity measured by RT-luminol.** Table shows the significant differences among treatments after two-way ANOVA analysis and Bonferroni post-tests. * = P< 0.05, ** = P< 0.01, *** = P< 0.001, ns= no significant difference. Unstim= Unstimulated, Mac= MacroGard® (100 µg/ml), Zym= Zymosan (100 µg/ml).

| | Minutes | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | 0-9 | 12 | 15 | 18-42 | 45 | 48 | 51-54 | 57-84 | 87 | 90 | 93 | 96 | 99 | 102-105 | 108 | 111 | 114 | 117 | 120 | 123-213 |
| Unstim NG-f-Mac NG-f | ns | ns | ** | *** | *** | *** | *** | *** | *** | *** | *** | *** | ** | * | ns | ns | ns | ns | ns | ns |
| Unstim NG-f-Zym NG-f | ns | ns | ** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | ** | * | * | ns |
| Zym NG-f-Mac NG-f | ns | ns | ns | ns | ns | ns | ** | *** | ** | ** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Unstim MQ-f-Mac MQ-f | ns | *** | *** | *** | *** | *** | *** | *** | *** | *** | ** | ** | * | ns | ns | ns | ns | ns | ns | ns |
| Unstim MQ-f-Zym MQ-f | ns | ns | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | ** | ** | * | ns | ns |
| Zym MQ-f-Mac MQ-f | ns | ns | ns | ns | * | ** | *** | * | ** | *** | ** | * | ns | ns | ns | ns | ns | ns | ns | ns |

# PAPER II

# β-glucan enriched bath directly stimulates the wound healing process in common carp (*Cyprinus carpio* L.)

**Przybylska DA[1], Schmidt JG[1], Vera-Jimenez NI[1], Steinhagen D[2] and Nielsen ME[1]**

**Address correspondence and reprint requests to** Nielsen ME. DTU Food, National Food Institute, Biological Quality Research Group, Søltofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark. E-mail address: mice@food.dtu.dk

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Short communication

## β-glucan enriched bath directly stimulates the wound healing process in common carp (*Cyprinus carpio* L.)

D.A. Przybylska-Diaz [a], J.G. Schmidt [a], N.I. Vera-Jiménez [a], D. Steinhagen [b], M.E. Nielsen [a,*]

[a] *DTU Food, National Food Institute, Biological Quality Research Group, Mørkhøj Bygade 19, DK 2860 Søborg, Denmark*
[b] *Fish Disease Research Unit, Centre of Infectious Diseases, University of Veterinary Medicine Hanover, Bünteweg 17, D-30559 Hanover, Germany*

**Abstract**

Wound healing is a complex and well-organized process in which physiological factors and immune mechanisms are involved. A number of different immune modulators have been found to enhance the non-specific defence system in vertebrates, among which β-glucans are the most powerful and extensively investigated.

The aim of the present study was to investigate the biological impact of two different commercially available β-glucan containing products on the wound healing process in carp. Throughout a two week experiment fish were kept either untreated (control), or in water supplemented with the two different types of β-glucans. The wound healing process was monitored using a multispectral visualisation system. The correlation between wound closure and immune response was investigated by measuring the gene expression patterns of IL-1β, IL-6, IL-8 and Muc5b, and measurement of production of radical oxygen species. PAMPs/DAMPs stimulation caused by the wounding and or β-glucans resulted in an inflammatory response by activating IL-1β, IL-6 and IL-8 and differences in the expression pattern were seen depending on stimuli. IL-1β, IL-6 and IL-8 were activated in all wounds regardless of treatment. Expression of all three interleukins was highly up regulated in control wounded muscle already at day 1 post-wounding and decreased at subsequent time-points. The reverse was the case with control wounded skin, where expression increased from day 1 through day 14. The results for the β-glucan treated wounds were more complex. The images showed significantly faster wound contraction in both treated groups compared to the control. The obtained results clearly demonstrated that a β-glucan enriched bath promotes the closure of wounds in common carp and induce a local change in cytokine expression.

**Keywords**: carp, *Cyprinus carpio* L., β-glucan, wound healing, gene expression

## 1. Introduction

Skin, together with the mucus, forms the first line of defence against pathogens and is an essential protective barrier in aquatic organisms. Mucus can entrap foreign particles and microorganisms, before they can interact with the epithelial surface and cause damage [1]. The main mucus components are water and large, highly glycosylated glycoproteins called mucins. Mucins and mucus have rheological properties (viscosity and elasticity), which are important for their physiological function [2]. Based on biochemical characterisation, 19 mucin genes have been identified in humans and gene structure as well as their role in the infection process has been described [3-5]. To date, only two mucin genes, Muc2c and Muc5b, have been cloned and sequenced in fish [6].

Wounds in fish can be caused by pathogens such as ectoparasites or Gram-negative bacteria, as well as by physical trauma [7-9]. In vertebrates reduced skin integrity or mucus production facilitates entry of pathogens into the underlying tissue and vascular system, therefore rapid reaction at the wound site is essential [10]. Wound healing is a complex and well-organized process which can be roughly divided into three overlapping phases: inflammation, tissue formation and remodeling, in which blood cells, soluble mediators, resident cells (fibroblasts, endothelial cells, goblet cells) and extracellular matrix components are involved [10-12].

The immunomodulatory effect of β-glucans has been shown in studies on several taxonomic groups including fish [13-18]. These naturally occurring polysaccharides differ in length, molecular weight, extent of branching and bioactivity, and in vertebrates trigger different biological effects [19-24]. β-glucans have been shown to accelerate the wound healing process in mammals, however such effect remains to be proven in fish [25, 26].

β-glucans are pathogen-associated molecular pattern molecules (PAMPs) and are recognized by pattern recognition receptors (PRRs) such as toll-like receptors (TLR) or NOD-like receptors (NLRs), and activate transcription of pro-inflammatory genes [27]. Furthermore, PRR signaling can also be activated by “danger signals” – damage associated molecular patterns (DAMPs), which are endogenous molecules such as nucleic acids, high-mobility group box 1-protein or heat shock proteins released from cells during tissue damage, or hyaluronan products degraded during said process [28, 29]. Hence, introducing PAMPs and DAMPs simultaneously may have a combined impact [30].

Mammalian models have shown that in response to PAMPs and DAMPs, numerous cytokines are secreted and subsequently, will guide and selectively activate leukocyte subsets [12]. Some cytokines have shown to be highly important during wound healing. Mammalian interleukin 1β (IL-1β) is strongly up-regulated during the inflammatory phase of healing [31, 32]. The main IL-1β sources are polymorphonuclear leukocytes (constitute nearly 50% of all cells at the wound site in first few days post-wounding) and macrophages, as well as some resident cells [12, 31]. In addition, human recombinant IL-1β is reported to affect mucus release in mice [33]. Interleukin 8 (IL-8) is a known regulator of neutrophil trafficking and augments angiogenesis in mammals [12, 32, 34-36]. Carp possess two CXCL8 lineages, which appear to be functional homologs to mammalian IL-8 [37]. Expression of IL-8 in mammals is correlated with interleukin 6 (IL-6) [38]. IL-6 is produced and secreted by macrophages, neutrophils, as well as resident cells [12, 31]. Experiments on diabetic or IL-6 knockout mice have shown a reduction in neutrophil and macrophage number, as well as a decrease in collagen production and deposition [39, 40]. Orthologues of the mammalian IL-6 have been identified in fish [41-44]. Moreover, PAMPs and DAMPs induce production and secretion of hydrolases, complement components, and reactive oxygen and nitrogen species (ROS and RNS) by macrophages in vertebrates [45-48].

In the present study, we have examined the direct biological effect of β-glucan enriched products on the wound healing process in common carp in a controlled environment. This effect has been verified by monitoring visual wound contraction dynamics and gene expression patterns of three pro-inflammatory cytokines: IL-1β, IL-6 and IL-8, as well as measurement of ROS production. Additionally, as Muc5b is the major gel-forming mucin in the protective mucous matrix of mammals, its expression was investigated [49].

## 2. Materials and methods

### 2.1 Animals

One-year-old common carp (*Cyprinus carpio* L., R3xR8, WUR, The Netherlands) were kept in 30 L aquariums filled with tap water adjusted to 21°C and fed a commercial carp feed (Trouvit, Nutreco) at 2% of their estimated body weight per day. Fish were exposed to a 12/12 light/dark cycle. The fish were divided into six groups (Table 1).

**Table 1.** Experimental groups.

| | | Water Condition | | |
|---|---|---|---|---|
| | | Control | MacroGard | 6.3 kDa fiber |
| Wounding | No wound | 12 | 12 | 12 |
| | 5 mm Biopsy | 12 | 12 | 12 |

## 2.2 Preparation of β-glucans

For stock solution preparation, 0.5 g of MacroGard (yeast, >60% pure, Biorigin, Brasil) or 6.3 kDa fiber (oat, 50% pure, Scan Oat, Sweden) was dissolved in 500 ml MilliQ water. In order to dissolve the soluble β-glucan fractions, solutions of both products were stirred for 1 h at 90°C and autoclaved (121°C, 15 min, 1 atm). In the experimental setup, water was changed daily and the β-glucan product concentrations were adjusted to 0.1 μg/ml.

## 2.3 Wounding and sampling procedure

Carp were anaesthetised by immersion in 0.01% benzocaine (VetPharm, Belgium). 5 mm Ø biopsy punches (Miltex, Inc, USA, Figure 1.) were used to mechanically damage areas of the skin and musculature (left side, above the lateral line, three wounds per fish). At day one, three and fourteen post-wounding four individuals from each group were anaesthetised and, using 8 mm biopsy punches, the following tissue samples from the edges of the wounds were collected: 1. Muscle tissue from the wounded area; 2. Muscle tissue from a non-wounded area (right side); 3. Skin tissue from the wound area; 4. Skin tissue from non-wounded area (right side); 5. Muscle and skin tissue from all control fish (left side, Figure 1). Samples were collected and immediately frozen in liquid nitrogen for further analysis. This experiment was conducted according to Danish legislation and by scientists accredited by the Federation of Laboratory Animal Science Associations (FELASA).

## 2.4 RNA isolation and cDNA synthesis for qPCR

The previously frozen in the liquid nitrogen samples (50 mg) were mixed with 500 µl of buffer (2-Mercaptoethanol in lysis buffer) and sonicated (30 s, pulse 2, amplitude 70 %, using a soVCX-130 sonicator CiAB, Sweden) as it is considered as more straightforward method than homogenization [50]. Following the manufacturer's protocol, RNA was isolated using GenElute Mammalian$^{TM}$ Total RNA Miniprep Kit (Sigma-Aldrich, Denmark) and subsequently treated with DNase-I (Sigma-Aldrich, Denmark) to remove any genomic DNA. RNA purity and quantity was determined by OD260/280 measurements on a NanoDrop$^{TM}$ 2000 spectrophotometer (Thermo Fisher Scientific, Germany). 400 ng of total RNA was primed with random hexamers and reverse transcribed using TaqMan® Reverse Transcription reagents (Applied Biosystems, Denmark) in a final volume of 20 µl. The synthesized cDNA was diluted 1:10 in MilliQ water and stored at -20 °C until further analysis.

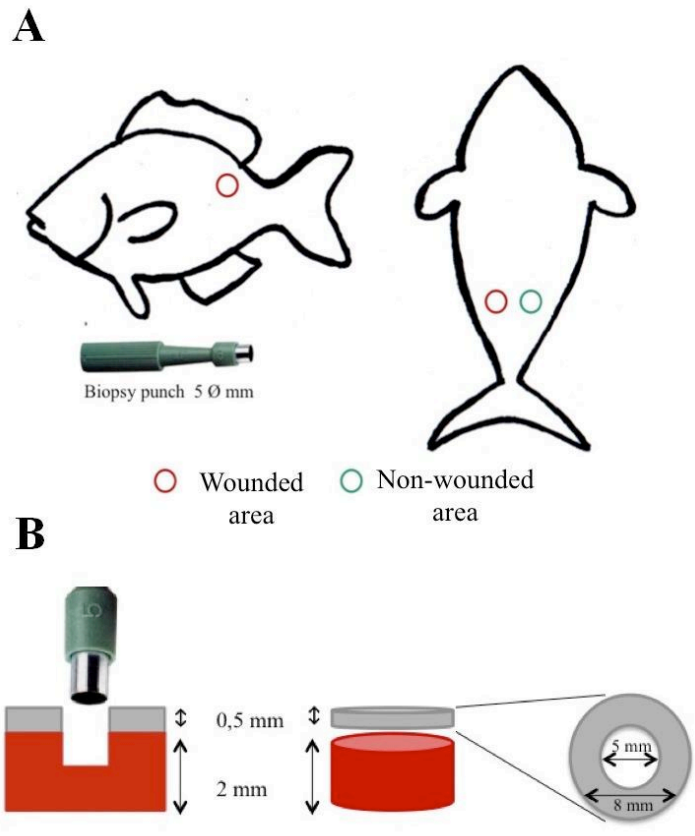



**Figure 1. Illustration of wounding (A) and sampling (B) procedures and biopsy punches**. Fish were wounded on the left side above the lateral line (red circle). During sampling, skin and muscle tissue were taken from the wounded site (red circle), as well as from non-wounded site (internal control, green circle).

## 2.5 Real-time quantitative PCR

Based on sequences deposited in the GenBank (refer to Table 2) and using the program Primer3, PCR primer sets specific for 40S, IL-1β, IL-6 and IL-8, were designed [51]. The mucin 5b (Muc5b) primer set was provided by Fish Disease Research Unit at the University of Veterinary Medicine, Hanover, Germany. The subunit S11 of the ribosomal gene 40S was selected as a reference gene based on previous work [7]. The assays for examined genes were run using SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma-Aldrich, Denmark). Quantitive RT-PCR was performed using a Stratagene MX3000P™ real-time PCR system (Integrated Sciences, Australia). Master mixes for each PCR run were prepared as follows: 12.5 µl Brilliant SYBR® Green JumpStart™ *Taq* ReadyMix™, 1 µl of each primer and 5.5 µl ultra pure water. Finally 5 µl of diluted cDNA was added to 20 µl of master mix. The cycling conditions for the reaction were: incubation step of 10 min at 95 °C, followed by 45 cycles of 95 °C for 30 s and 60 °C for 1 min. At the end of each reaction, DNA melting curve analysis was performed in order to confirm the specificity of the PCR products. All samples were run in triplicate, and for each primer set, control reactions without cDNA were included in order to detect any non-specific amplification. The expression results were analyzed using the $-2^{-\Delta\Delta Ct}$ method [52]. Data are shown as fold expression relative to non-wounded internal control site, and the analysis was carried out using Mx ProTM qPCR software (Integrated Sciences, Australia). The threshold values (Ct) were determined manually for each run.

**Table 2.** Sequences of primers used for real-time PCR.

| Gene | Primer | Sequence (5' - 3') | GeneBank acc. No. |
|---|---|---|---|
| 40S | Forward | GTTGAAGGAAGTGGCAAGGA | AB012087 |
| | Reverse | AGAATACGGCCTCTGATGGA | |
| IL-1β | Forward | AAGGAGGCCAGTGGCTCTGT | AJ245635 |
| | Reverse | CCTGAAGAAGAGGAGGCTGTCA | |
| IL-6 | Forward | CCGCACATGAAGACAGTGAT | AY102632 |
| | Reverse | GGGTATATTTGGCTGCAGGA | |
| IL-8 | Forward | TGGAGCTCTTCCCTCCAAG | EU011243 |
| | Reverse | AGGGTGCAGTAGGGTCCAG | |
| Muc5b | Forward | CAGCCCTCTTCCTCTTTCATC | JF343438 |
| | Reverse | CCACTCATCTTTCCTTTCTCTTC | |

## 2.6 Isolation of head-kidney (HK) cells and measurement of respiratory burst

Anaesthetised fish were bled from the caudal vein. Both head-kidneys (HK) were excised and placed on a 100 µm nylon cell strainer (BD Falcon, New Jersey, USA). A cell suspension was obtained by pressing the head-kidneys with a plunger through the cell strainer, and rinsing them with phenol red-free Hank's balanced salt solution with 0.1 % gelatine (g-HBSS). The HK cells were counted using a Bürker chamber. Cell viability was assessed by Trypan exclusion and cell concentration was adjusted to $3x10^6$ HK cells/ml in g-HBSS. Subsequently, the respiratory burst activity was measured using luminol-amplified chemiluminescence, modified from the protocol described by Vernho *et al*. (2005) [53]. To elicit the respiratory burst response, *Aeromonas hydrophila* were incubated overnight. The bacteria was washed twice with HBSS, $OD_{600}$ was set to 0.5, and 50 µl was added to each well in a white 96-well plate (Sigma-Aldrich P8616, Denmark). Next, a mixture of 40 µl of 10mM luminol in 0.2 M borate buffer (pH 9.0) and 100 µl of HK cells were added. The total well volume was adjusted with g-HBSS to 300 µl. The chemiluminescence emission from the HK cells was measured with a luminometer (Synergy2, Biotek) every 3 minutes for 210 min. at 26°C. Results are expressed as the integral of the relative light units (RLU) recorded by the luminometer (Max RLU).

## 2.7 Visual analysis of wound closure

Prior to sampling of the head kidney, skin and muscle, images of the wounds were acquired using a VideometerLab (Videometer A/S, Hørsholm, Denmark). The fish were placed in a special container in order to exclude the ambient light as the VideometerLab provides diffuse illumination from light-emitting diodes to capture 20 images with unique spectra between ultra violet and near infra red of the electromagnetic spectrum. The multispectral imaging facilitated the visual detection of the wound edge. The distance from the camera to the right (intact) side of the fish was kept constant. Differences in width of individual carp could affect the image capture area of the wound. However, given the long distance from the camera to the subject (~40cm) and narrow size range of the fish, this had no measurable effect on wound size estimations. The wound edges on day 14 post-wounding were outlined and the resulting open wound area determined using a script in MATLAB (The MathWorks Inc., Natick, MA, USA).

## 2.8 Statistics

A non-parametric Mann-Whitney test was used to compare results from gene expression and respiratory burst. Differences between groups were regarded as significant at $p < 0.05$. Results from visual wound closure were tested with a two-way ANOVA using the Prism software, version 4.03 (Macintosh, GraphPad Inc., La Jolla, CA, USA).

# 3. Results

## 3.1 The visual healing of the wounds

At day 14 post-wounding, the wound size was significantly ($p < 0.01$) reduced in both β-glucan treated groups in comparison to control fish, but significantly more in the MacroGard-treated group. Figure 3 shows the differences in open wound area 14 days post-wounding from the experimental groups (Figure 2. and 3.).

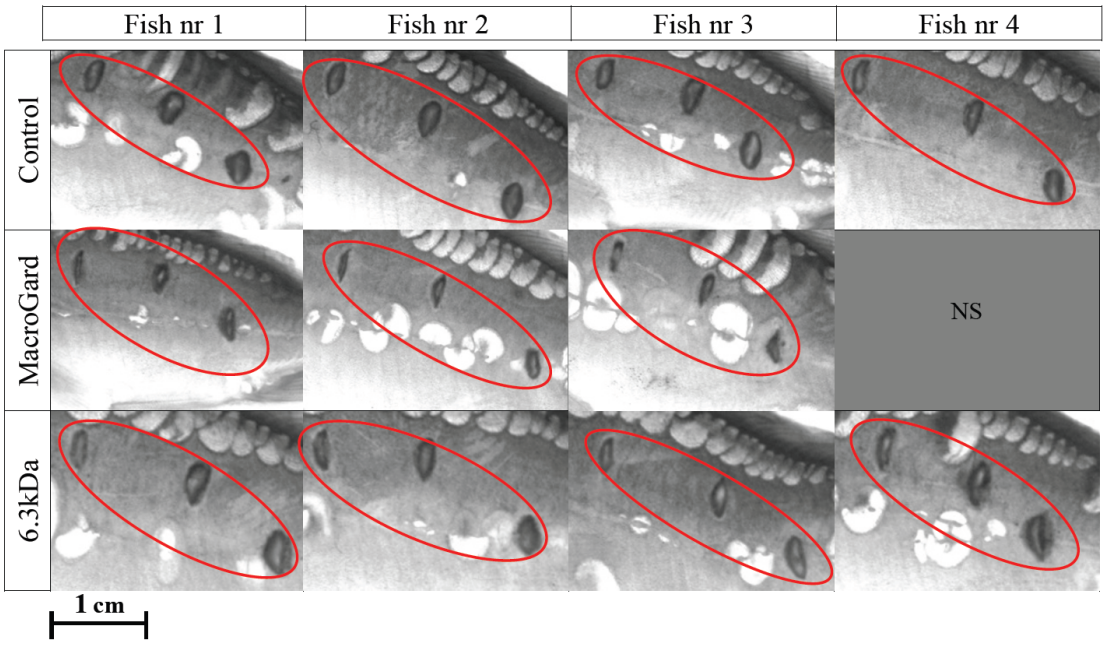



**Figure 2. Images of the wounded area fourteen days post-wounding acquired with the VideometerLab.** Top row – control, middle row – MacroGard, botton row – 6.3 kDa oat fiber. NS – no sample.

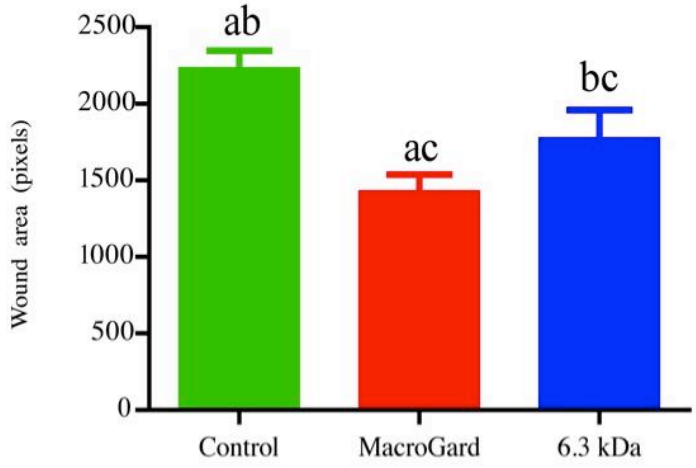



**Figure 3. Statistical representation of the wound sizes fourteen days post-wounding from wounded-control, wounded-MacroGard and wounded-6.3 kDa groups.** Bars show mean values (pixels) + SD of n=4 (n=3 14 days in wounded-MacroGard group). "a" and "b" depict statistical significance ($p < 0.01$) between control and MacroGard groups, and control and 6.3 kDa groups subsequently. "c" depicts statistical significance ($p < 0.05$) between MacroGard and 6.3 kDa groups.

## 3.2 Gene expression during wound healing process

No significant changes were observed in non-wounded groups.

### 3.2.1 Interleukin 1β (IL-1β)

In skin collected from the W-C group, IL-1β showed a tendency to increase over time (Figure 4A). Muscle samples taken one day post-wounding showed a high and significant up-regulation ($p < 0.05$) (Figure 4B).

In skin collected from the W-MG group, a significant up-regulation ($p < 0.05$) three days post-wounding was observed (Figure 4A). No significant changes in muscle were seen (Figure 4B).

In skin collected from the W-6.3 group, IL-1β expression was low but significant ($p < 0.05$) one day post-wounding (Figure 4A). In muscle, high and significant up-regulation ($p < 0.05$) was observed one day post-wounding (Figure 4B).

### 3.2.2 Interleukin 6 (IL-6)

In skin collected from the W-C group, IL-6 showed a tendency for up-regulation three and fourteen days post-wounding (Figure 4C). In muscle, significant up-regulation ($p < 0.05$) was noted on day one post-wounding (Figure 4D).

In skin collected from W-MG fish, significant IL-6 up-regulation ($p < 0.05$) was seen three days post-wounding (Figure 4C). In musculature tissue, no significant changes were seen (Figure 4D).

In skin collected from W-6.3 fish, a tendency for up-regulation was observed (Figure 4C). In muscle, a high and significant up-regulation ($p < 0.05$) was detected one day post-wounding (Figure 4D).

### 3.2.3 Interleukin 8 (IL-8)

In skin collected from the W-C group, three and fourteen days post-wounding, a high and significant up-regulation ($p < 0.01$) of IL-8 was observed (Figure 4E). In muscle, a high and significant up-regulation ($p < 0.01$) was noted one day post-wounding (Figure 4F).

In skin collected from W-MG fish, IL-8 showed a tendency to be down-regulated over time (Figure 4E). In muscle, a high and significant up-regulation ($p < 0.01$) was detected three days post-wounding (Figure 4F).

In skin collected from W-6.3 fish, a tendency for IL-8 up-regulation was seen fourteen days post-wounding (Figure 4E). In muscle, significant up-regulation ($p < 0.05$) was detected three days post-wounding (Figure 4F).

### 3.2.4 Mucin 5b (Muc5b)

In skin collected from W-C fish, a significant up-regulation ($p < 0.05$) of Muc5b was observed fourteen days post-wounding (Figure 4G). In muscle samples taken fourteen days post-wounding, a tendency for up-regulation was observed (Figure 4H).

In skin collected from the W-MG group, no significant differences in Muc5b expression were observed between wounded and non-wounded sides (Figure 4G). In muscle, a tendency for up-regulation was noted fourteen days post-wounding (Figure 4H).

In skin collected from the W-6.3 group, a significant up-regulation ($p < 0.05$) in Muc5b expression was observed three days post-wounding with tendency to be up-regulated fourteen days post-wounding (Figure 4G). In muscle a tendency for up-regulation was seen fourteen days post-wounding (Figure 4H).

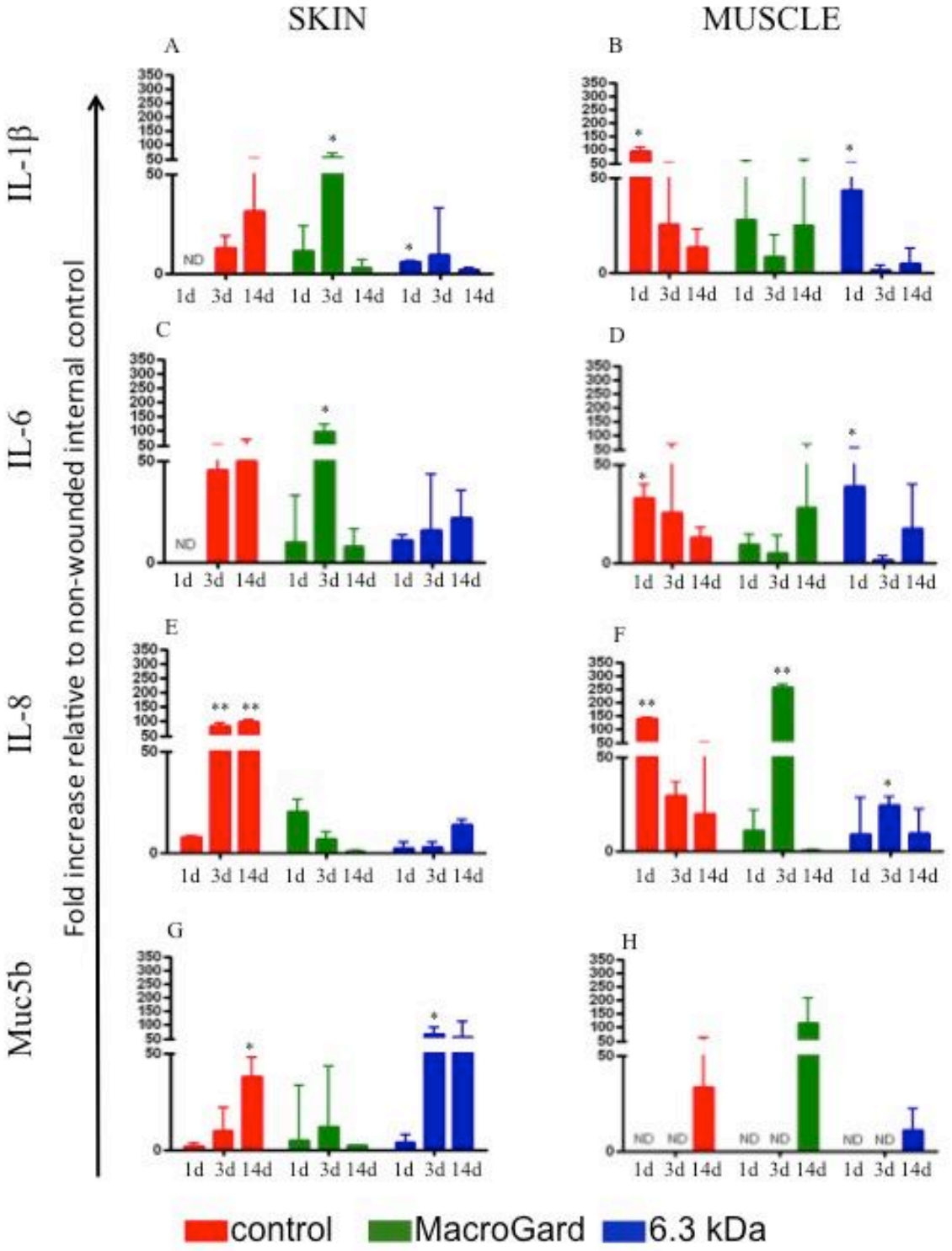
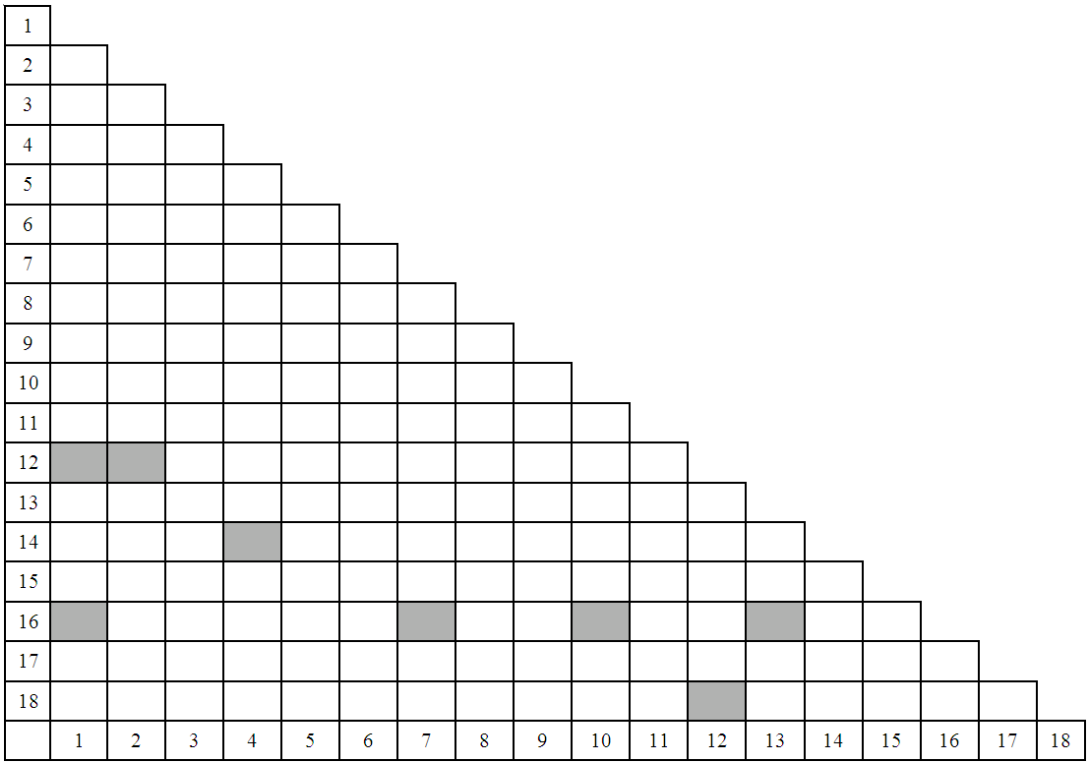



**Figure 4. Quantitative real-time PCR for mechanically wounded fish.** Expression of the genes interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8) and mucin 5b (Muc5b) is shown in skin (A, C, E, F) and muscle (B, D, F, H) of common carp. Results are obtained by qPCR and expressed relative to the internal control at each time point. The data are normalized relative to 40S and analyzed using the -2 –ΔΔCt method. Bars represent fold expression + SD relative to non-wounded side of n=4 (n=3 14 days in wounded-MacroGard group). cnt – control; ND – not detected. * Depicts statistical significance between the wounded site and internal control site (*$p < 0.05$; **$p < 0.01$).

## 3.3 Measurement of respiratory burst

Some significant differences between groups was observed (grey color), however they were inconsistent with no regular pattern. The absence of marked differences on ROS production in head kidney leukocytes was a sign of a localized immune response (refer to Table 3).

**Table 3.** Respiratory burst significant differences within or among treatments, measured by luminol-amplified chemiluminescence of carp head kidney (HK) leukocytes. The gray color indicates the only combinations with a significant difference (p value= 0.0286). 1) non-wounded control (1dpw), 2) non-wounded MacroGard (1dpw), 3) non-wounded 6.3 kDa (1dpw), 4) non-wounded control (3dpw), 5) non-wounded MacroGard (3dpw), 6) non-wounded 6.3 kDa (3dpw), 7) non-wounded control (14dpw), 8) non-wounded MacroGard (14dpw), 9) non-wounded 6.3 kDa (14dpw), 10) wounded-control (1dpw), 11) wounded-MacroGard (1dpw), 12) wounded-6.3 kDa (1dpw), 13) wounded-control (3dpw), 14) wounded-MacroGard (3dpw), 15) wounded-6.3 kDa (3dpw), 16) wounded-Control (14dpw), 17) wounded-MacroGard (14dpw), 18) wounded-6.3 kDa (14dpw).




## 5.Discussion

Fish mucosal immunity is an extensively investigated area [54]. In contrast to terrestrial vertebrates fish skin is not keratinised and is hence metabolically active [55, 56]. Following wounding, DAMPs activate mucosal immune cells as well as epithelial resident cells, e.g. in vertebrates, fibroblasts have been shown to possess immune regulation capabilities [57-59]. Several fibroblast cell lines have been established and characterised in fish, and Ingerslev *et al.,* (2010) have reported that fish fibroblasts are susceptible to DAMPs [57, 60, 61]. In the

present study, high up-regulation of IL-1β and IL-6 in skin samples from W-MG fish three days post-wounding could be explained by a boost of the immune-like performance from fibroblasts, elicited by the presence of DAMPs/PAMPs. Wei *et al*., (2002) presented the first evidence that glucans directly stimulate human fibroblasts [62]. Therefore fish fibroblasts, which could be activated in a similar way to immune competent cells, might directly stimulate the resident cells present in skin, such as epithelial, resident macrophages and lymphocytes, and amplify cytokine profiles leading to tissue regeneration. In contrast, fish Malpighian cells, regarded as the counterpart of the mammalian keratin-containing keratinocyte, which actively participate in wound healing process in fish, were shown not to react *in vitro* to yeast cells obtained from *Saccharomyces cerevisiae* [63].

Few studies have emphasized the role of goblet cells and mucus in wound healing process in mammals [11, 64]. Goblet cell differentiation and function in mammalian systems are affected by mucosal immunity, and gel-forming mucins can be regulated by different cytokines or exogenous factors [2, 6, 33, 65, 66]. Van der Marel *et al*., (2012) have shown that Muc5b is exclusively expressed in skin and gills of common carp, therefore the Muc5b expression was expected to change in carp subjected to β-glucan bath [6]. High Muc5b up-regulation three and fourteen days post-wounding was, however, not restricted to the wound area but was a general response of the skin mucosa to the wounding. It is in agreement with work presented by van der Marel *et al*. (2010) where water with increased bacterial load did not induce clinical symptoms in carp, however a rapid skin mucosal response was observed even if the bacteria involved were considered to be non-pathogenic [67]. Moreover, high local inflammation and IL-1β expression could hamper Muc5b expression at the site of wounding which is in accordance with work presented by Cohan *et al*. (1991). This could also explain why Muc5b expression was not higher in the MacroGard treated group as would have been expected. However, although Muc5b in vertebrates is one of the largest gel-forming glycoprotein in the body, individual cells may differ in mucin composition [2, 49, 68]. Thus it cannot be excluded that β-glucan supplemented bathing induced expression of other mucins in the skin of carp which were not included in the present study. According to work presented by van der Marel *et al.* (2012), Muc5b was exclusively expressed in skin and gills of common carp [6]. However, in the present study, expression of Muc5b was detected in muscle samples collected fourteen days post-wounding. It was suggested by Cheng and Leblond (1974), and further by Paulus *et al*. (1993) and Kanter and Akpolat (2008), that columnar and goblet cells in mammals originate from a common stem cell located at the

crypt base [11, 69, 70]. If that is the case in fish model, Muc5b detected in muscle could be an effect of goblet/mucus cell migration toward newly differentiating muscle layers.

Tissue damage and release of danger signals such as RNA/DNA or heat shock proteins influence expression of pro-inflammatory cytokines [12, 27]. High levels of IL-1β, IL-6 and IL-8 cytokines one day post-wounding in muscle collected from the W-C group is likely to be the inflammatory response caused by the release of DAMPs. Interestingly, in skin samples from W-C fish, different patterns of pro-inflammatory cytokines expression has been noted. No or low IL-1β, IL-6 and IL-8 expression was detected one day post-wounding. However, gene expression was not investigated at earlier time points. It is possible that stored IL-1β, IL-6 and IL-8 have been secreted quickly (hours) in response to wounding and subsequently, these stores needed to be replenished. The temporally inversed pattern of gene expression in skin/muscle tissue has been seen in both β-glucan supplemented groups, with change in expression three days post-wounding. It is consistent with work done on rodents by Wolk and Danon (1985) [25]. According to these authors, the most pronounced differences in wound closure between glucan treated and control groups took place between 48 and 96 hours. All changes occurred earlier in the glucan treated group, and included proliferation and arrangement of fibroblasts as well as deposition of the collagenous matrix.

Yeast β-glucan is known as a powerful immune modulator and many studies have described its positive effect on various vertebrates [14-18, 25]. Bohn and BeMiller (1995) described β-glucans as biological response modifiers with activity correlated to the branching degree and size [24]. Accordingly, no biological activity/effect was detected in mammalian models when less branched glucans with molecular weights of 5 kDa-10 kDa were used. In contrast, Tanioka *et al*. (2011) have shown that barley-derived β-glucan (~2 kDa) stimulates maturation of mouse dendritic cells [71]. In addition, up-regulation (~ x30) of IL-6 expression in murine bone marrow cells has been seen when compared to treatment with 40-70 kDa barley β-glucan [71]. Present results show that both β-glucans, MacroGard and 6.3 kDa oat fiber, promote the wound healing process in common carp and therefore 6.3 kDa oat fiber can be considered bioactive.

To our knowledge, this is the first investigation of the biological effect of β-glucans on the wound healing process in fish. Furthermore it is the first experimental design that allows the open wounds to stay in direct and constant contact with the β-glucan supplementation during the entire trial. Many studies have described the positive effect of β-glucans on the fish

immune system [13-18]. However, the mechanism by which β-glucans enhance the wound closure remains unclear [14-18, 72]. Wolk and Danon (1985) have emphasized that application of glucans to promote wound healing in vertebrates would require an experimental model in which a wound on one side of the animal could be compared to a symmetrical wound on the other side of the same animal [25]. In our study, internal controls from non-wounded site of the wounded fish have been studied. This allowed us to eliminate external factors (e.g. temperature) and focus on obtaining more data about local and systemic response in carp during the wound closure process.

Overall, our results show that both β-glucans promote wound healing process in common carp when compared to control fish. We have concluded that bathing in β-glucans has direct positive effects on the wound closure in common carp and it can suggestible be related to high branching level due to fish being bathed in MacroGard supplemented water showed higher wound closure ratio in comparison to 6.3 kDa supplemented bath. We have shown the immunological and regenerative response following stimulation with β-glucans (PAMPs) and wounding (DAMPs) in controlled conditions without exposure to pathogens. PAMP/DAMP stimulation resulted in an inflammatory response by activating IL-1β, IL-6 and IL-8, and local differences in expression patterns depended on major stimuli: DAMPs or DAMPs/PAMPs combination. In addition, the absence of marked differences on the respiratory burst activity in head-kidney cells supports the notion of a local immune response at the site of wound.

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**PAPER III**

# Carp head kidney leukocytes display different patterns of oxygen radical production after stimulation with PAMPs and DAMPs

**N. I. Vera-Jiménez and M.E. Nielsen**

**Address correspondence and reprint requests to** Nielsen ME. DTU Food, National Food Institute, Biological Quality Research Group, Søltofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark. Telephone number: +45-45254906. E-mail address: mice@food.dtu.dk

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Carp head kidney leukocytes display different patterns of oxygen radical production after stimulation with PAMPs and DAMPs

N.I. Vera-Jimenez, M.E. Nielsen*

*DTU Food, National Food Institute, Division for Industrial Food Technology, Biological Quality Group, SøltoftsPlads, Building 221, DK-2800 Kgs. Lyngby, Denmark*

**Abstract**

Wound healing and tissue regeneration are essential mechanisms to ensure the survival and health of any organism. Despite this, only a few studies have been devoted to study tissue regeneration during wound healing in fish. Reactive oxygen species (ROS), in particular hydrogen peroxide, play an important dual role both for promoting tissue repair, but also for eradication of pathogens. This study aims at dissecting the contribution of PAMPs (using β-glucan) and DAMPs in the respiratory burst response of carp head kidney-derived leukocytes, and address their contribution to wound healing processes. Consistent with a pathogen eradication strategy, ROS responses to PAMP stimulation (β-glucan) was fast, vigorous and highly dominated by production of superoxide anion. In contrast, stimulation with DAMPs led to a slow, subtle but long-lasting production of oxygen radicals dominated by hydrogen peroxide. Using an *in vitro* model of scratch-wounded CCB fibroblast cell cultures and a novel PhotoID proliferation assay, stimulation with low and continuous levels of hydrogen peroxide (5 μM) led to a slight increase in the percentage of wound recovery and thus promoted wound closure. In contrast, high doses of hydrogen peroxide (300 μM) impaired fibroblast scratch-wound recovery and caused cell death. These results elucidate the capacity of hydrogen peroxide to influence the fate of tissue regeneration through the establishment of environments suitable for promoting either tissue regeneration or oxidative stress and thereby potential tissue damage. Direct *in vitro* stimulation with β-glucans did not impact fibroblast scratch-wound recovery, which further suggests that interaction with tissue-resident leukocytes or other components of the fish immune system are required to induce fibroblast proliferation and thus for the accelerated wound healing promoted by β-glucan stimulation.

**Keywords:** *PAMPs; DAMPs; Tissue regeneration; Respiratory burst kinetics; Common carp; β-glucans.*

## 1. Introduction

A normal healing response starts when the tissue is injured. Work on mammalian models has shown that damage initiates a cascade of events with a coordinated interaction of different cell types which are involved in several overlapping phases including coagulation, early/late inflammation, cell proliferation and tissue remodelling (Diegelmann and Evans, 2004, Schaffer and Barbul, 1998, Mescher and Neff, 2005, Werner, Krieg et al., 2007, Matsubayashi, Razzell et al., 2011). Conversely, in fish little is known about the interactions between the immune system and the later phases of the wound healing process, albeit the inflammatory phases have been studied (Gonzalez, Huising et al., 2007, Forlenza, Fink et al., 2011).

Detection of tissue damage in the host is accomplished by the immune system, which uses a series of receptors displayed by the immune cells to identify the nature of the injury and act towards its repair. Such receptors are able to discriminate between the scenery related with an open wound, (implicating the intrusion of pathogens in the injury), and those consistent with pathogen-free trauma (evoking sterile inflammation) (Schreml, Szeimies et al., 2010, Rock, Latz et al., 2010). The aforementioned receptors are able to distinguish the different injury sceneries based on molecular signals. For example, pathogens display series of specific molecular motifs called pathogen-associated molecular patterns (PAMPs), which are not found in the host. On the other hand, identification of pathogen-free trauma is accomplished by the recognition of damage-associated molecular pattern (DAMPs), molecules from the host which in healthy cells are contained in the cells and hidden from the immune cells (Lotze, Zeh et al., 2007). Both situations activate signalling pathways leading to the destruction and elimination of the pathogens and infected or damaged cells (Bianchi, 2007).

The involvement of compounds such as reactive oxygen species (ROS), nitric oxide (NO), cytokines, chemokines and growth factors during pathogen eradication and tissue regeneration have been studied (Bianchi, 2007, Werner and Grose, 2003). Immune cells like neutrophils, macrophages and lymphocytes are known to produce these molecules after they encounter pathogens or sense DAMPs (Rock, Latz et al., 2010, Lotze, Zeh et al., 2007, Werner and Grose, 2003, Witte and Barbul, 2002, Kanta, 2011, Sen, 2009). However, there is growing evidence that non-immune cells such as fibroblasts, smooth muscle cells, endothelial cells and keratinocytes are able to reproduce this signal molecules, although to a lesser degree (Werner, Krieg et al., 2007, Witte and Barbul, 2002, Finkel, 1999, Schaffer, Efron et al.,

1997). An extraordinary coordination for the quantities and timing in the production of these molecules is needed, and exerts an important impact in the outcome of wound healing (Diegelmann and Evans, 2004, Wallach-Dayan, Golan-Gerstl et al., 2007). For example, while ROS in low concentrations (1-25μM) function as a second messengers and promote cell proliferation, at higher concentrations (25-50μM) have microbicidal effects, and in excessive amounts (>50μM) can impair cell growth and cause apoptosis (Kanta, 2011).

Fibroblasts are recognized as a link between the immune system and the tissue regeneration machinery, since they are involved in the production of collagen and other extracellular matrix components (ECM), and are major regulators of inflammation during wound healing (Diegelmann and Evans, 2004, Ingerslev, Ossum et al., 2010, Trengove, Stacey et al., 1999). Cytokine profiles produced by leukocyte subsets can influence macrophages and fibroblast to reproduce the same cytokine profile, which can lead to tissue repair with normal architecture or favours fibroblast activation tower higher collagen production rates and fibrogenesis (Mescher and Neff, 2005). Thus, the cellular responses of leukocytes and fibroblasts, and their interactions during wound healing are of great importance for the understanding of tissue regeneration processes.

β-glucans are glucose polysaccharides present in the cell wall of yeast, mushrooms, bacteria, algae and plants (Dalmo and Bøgwald, 2008). In mammalian systems, the immune stimulatory activity of β-glucans, especially the enhancement of tissue regeneration processes has been reported (Brown and Gordon, 2003, Reynolds, Kastello et al., 1980, Son, Bae et al., 2005, Chen and Seviour, 2007). Such immune stimulatory effects have also been described in several fish species (Dalmo and Bøgwald, 2008) however information on the effect of β-glucan in tissue regeneration processes in fish is scarce. We have previously reported that β-glucan enriched bath promotes wound closure in common carp ((Przybylska, Schmidt et al., 2013)), however the mechanisms through which β-glucan accelerates the wound healing process remains to be understood.

Since leukocytes, reactive oxygen species and fibroblasts have been shown to play a key role during tissue regeneration, this paper studies the oxygen radical response of carp head kidney-derived macrophages to DAMPs and β-glucans. Furthermore, the direct effect of β-glucans and hydrogen peroxide on carp fibroblast scratch-wound recovery is discussed.

## 2. Materials and Methods

### *2.1 Fish*

European Common carp (*Cyprinus carpio carpio*) were obtained from the central fish facility 'De Haar-vissen' (Wageningen, The Netherlands). R3xR8 carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and the Polish origin (R3) (Irnazarow, 1995). The fish used ranged between 50 to 100 g and were kept at 23°C (±1°C) with 12:12 h light: dark photoperiod.

### *2.2 Carp head kidney leukocyte isolation and culture:*

Head kidney leukocyte (HKL) isolation and culture were performed based on the protocol described by Joerink et al. (Joerink, Ribeiro et al., 2006). Briefly, carps were euthanized using an overdose of MS-222 (100 mg/l) and bled from the caudal vein. Head-kidneys were aseptically excised and placed in a 100 µm nylon cell strainer (BD Falcon). Head kidneys were gently pressed with a plunger through a 100-µm sterile nylon mesh and rinsed with homogenization buffer (incomplete-NMGFL-15 medium containing 50 U/ml penicillin (Sigma-Aldrich, Cat nr.P4458), 50 µg/ml streptomycin (Sigma-Aldrich, Cat nr.P4458), and 20 U/ml heparin (Leo Pharma)). Cell isolation was performed using a non-continuous percoll gradient (Sigma-Aldrich, Cat nr. P4937). After 25 minutes centrifugation at 800g, the cells present in the 1,02-1,08 interface were collected, washed two times with incomplete-NMGFL-15 medium and resuspended in 1ml complete-NMGFL-15 medium (incomplete-NMGFL-15 medium supplemented with 10% bovine calf serum (Sigma-Aldrich, Cat nr.F2442) and 5% heat-inactivated pooled carp serum). Cell viability was assessed by Trypan Blue exclusion (Sigma-Aldrich, Cat nr.T8154). Cell cultures were initiated by seeding $1.75 \times 10^7$ HKL in a 75-cm2 culture flask (Sigma-Aldrich, Cat nr. CLS430641) containing 20 ml of complete-NMGFL-15 medium with 50 U/ml of penicillin and 50 µg/ml streptomycin. Cells were incubated at 27°C with 5% CO2 during 6 days. Subsequently, HKL were harvested by placing the flasks on ice for 30 min and gentle scraping.

### *2.3 Fibroblast cell culture:*

CCB fibroblasts were kindly provided by Dr Dieter Steinhagen (Fish Disease Research Unit, School of Veterinary Medicine Hannover). Cells were grown at 27°C with 5% $CO_2$ in

Minimum essential medium eagle (Sigma-Aldrich, Cat nr.M4655) supplemented with 10% bovine calf serum (Sigma-Aldrich, Cat nr.F2442), 0.2% MEM non-essential amino acids solution (Sigma-Aldrich, Cat nr.M7145), 1% Penicillin-Streptomycin (Sigma-Aldrich, Cat nr.P4458) and 3.5 D-(+) Glucose solution 10% (Sigma-Aldrich, Cat nr.G8644). Cultures were split (1:3) when they reached 80% confluence using Trypsin-EDTA 0.25% solution (Sigma-Aldrich, Cat nr.T4049), medium was renewed every 3 days.

*2.4 Preparation of β-glucans*

During this study two different β-glucans were used: MacroGard®, which is a bakers' yeast extract containing a 60% purified fraction of 1,3/1,6 β-glucan (Siwicki, Zakęś et al., 2010), and Zymosan a glucan molecule with repeating glucose units connected by β-1,3 glycosidic linkages. Both β-glucans have shown to trigger respiratory burst in different cell populations and several fish species (Dalmo and Bøgwald, 2008, Novak and Vetvicka, 2008).

Stock solutions of MacroGard® (Biorigin) and Zymosan A (Sigma-Aldrich, Cat nr. Z4250) were prepared in milliQ water (PURELAB Ultra, Elga) at 20 mg/ml and 10 mg/ml respectively and sonicated twice during 30 seconds using power 6 of a Brandson sonifier 250. Subsequently, the sonicated solutions were pasteurized using a thermoblock at 80°C during 20 minutes.

*2.5 Preparation of Danger-associated molecular patterns (DAMPs)*

CCB fibroblasts were harvested with Trypsin-EDTA 0.25% solution and rinse twice, counted and resuspended in 8 ml of Dubbelco's phosphate buffered saline (dPBS, Sigma-Aldrich, Cat nr. D8537). Subsequently, cells were sonicated with 3 pulses of 5 seconds (130Watts / 20 Khz / 70% amplification) using an alcohol sterilized MSE soniprep 150 sonicator (Sanyo), and the solution was centrifuged at 14000g, 4°C during 10 minutes. The DAMPs supernatant was aliquot and stored at -80°C. The cell debris, referred in this paper as DAMPs pellet, was resuspended in 8 ml of dPBS, aliquot and stored at -80°C.

*2.6 Measurement of reactive oxygen species (ROS):*

The measurement of ROS was performed using the RT-luminol assay as explained previously (Allen, Stjernholm et al., 1972, Vera-Jimenez, Pietretti et al., 2013). In general, white 96-well plates (Corning®, Cat nr. 3917) were prepared containing 40 µl of luminol (10mM, Sigma-Aldrich, Cat nr.A8511) in 0.2 M borate buffer (pH 9.0) and $0.5X10^6$ HKL/well. As a stimulus

Zymosan (100 µg/ml), the DAMPs supernatant from $0.5X10^6$ CCB fibroblast or DAMPs pellet from $0.5X10^6$ CCB fibroblast were used. To identify the type of reactive oxygen species produced after stimulation of Zymosan and DAMPs, some of the wells were treated with either catalase (Cat, 300 U/ml), to provoke the dismutation of hydrogen peroxide to water and oxygen (Jones, 1982), or with superoxide dismutase (SOD, 250 U/ml) to catalyze the dismutation of superoxide to hydrogen peroxide and oxygen (McCord and Fridovich, 1969). The final volume of each well was always 300 µl, and the chemiluminescence emission from the HKL was measured with a luminometer (synergy2, Biotek) every 3 minutes at 26°C during 210 minutes. The results are expressed either as the kinetic curve of relative light units (RLU) recorded during 210 minutes, or as the integral of the relative light units (Max RLU) recorded by the luminometer between 0 and 210 minutes.

*2.7 CCB Fibroblast scratch-wound assay:*

CCB fibroblasts were plated in 6-well cell culture plates at an initial density of $0.5x10^6$ cells/well, 1 day before wounding. Cells were incubated at 27°C with 5% $CO_2$ as explained in section 2.3. Linear scratches were made in the monolayers with a pipette tip, producing a wound of ≈700 µm. The medium was renewed and the cultures stimulated with 100 µg/ml Zymosan, 100µg/ml MacroGard®, 5, 10 or 300 µM Hydrogen peroxide (Merk, Cat nr. ZU74080709), DAMPs supernatant from $0.5X10^6$ CCB fibroblast or left untreated. The medium was changed daily and the stimulus added once per day until the wound was closed. To follow wound closure microscopy pictures were taken at fixed coordinates in each well at 4X magnification objective (Olympus UPlanFLN, 4x/0.13PhL) using an inverted microscope (Olympus IX51) with an attached camera (Olympus XC50). Images were made immediately after wounding, at day 1, 2, and 3 post wounding. The wound edges of each picture were manually delineated using the software Adobe Photoshop CS4, and the pixels representing the wound area calculated with the image analysis toolbox in MatLab (2010). Results are expressed as percentage of wound recovery.

*2.8 Statistical analysis:*

The software GraphPad Prism (version 4.03) was used for statistical work. Statistical analysis was performed by Man Whitney test. In experiments involving ROS kinetics a two-way ANOVA analysis and Bonferroni post-tests were used. $P<0.05$ was considered to be statistically significant.

## 3. Results

### *3.1. Carp head-kidney leukocytes (HKL) display different oxygen radical production kinetics after β-glucan and DAMPs stimulation.*

The ROS production of carp HKL after stimulation with Zymosan, DAMPs supernatant or DAMPs debris was measured using RT-luminol assay. Stimulation with Zymosan generated a fast and vigorous oxygen radical response with a clear peak of 4000 RLU magnitude, 30 minutes post-stimulation (See fig. 1). The peak was followed by a uniform reduction of magnitude until the end of the measurement; significant differences are described in Table 1. Stimulation of HKL with DAMPs supernatant showed lower magnitudes of oxygen radical production when compared to the control during the first 30 minutes post-stimulation (See fig. 1). The highest magnitude of DAMP supernatant -elicited oxygen radical production (2459 RLU) was reached 147 minutes after stimulation, significant differences are described in Table 1. DAMPs debris stimulation of carp HKL followed the same pattern than the one described for DAMPs supernatant, reaching the highest magnitude(1525 RLU) 117 minutes post-stimulation. No significant differences were found in DAMP debris-elicited oxygen radical response in respect to unstimulated HKL (Fig.1, Table 1).

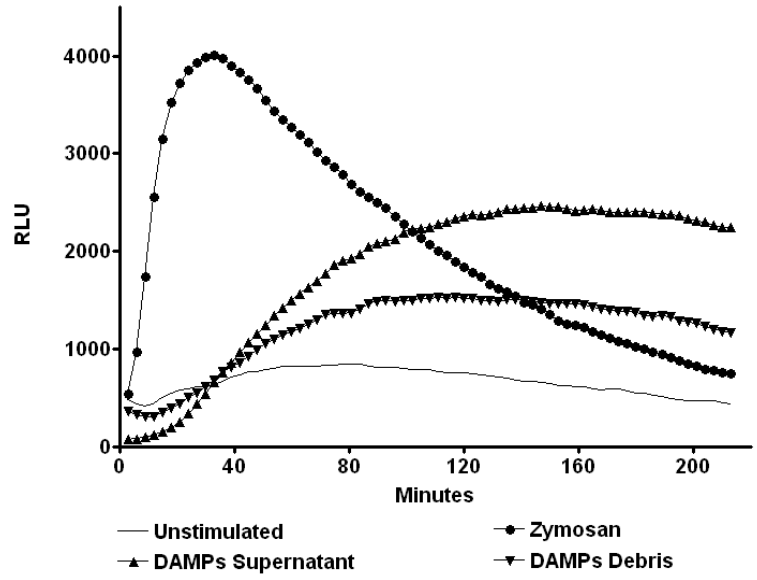


**Figure 1. Carp head-kidney leukocytes (HKL) display different oxygen radical production kinetics after β-glucan and DAMPs stimulation.** Carp HKL were stimulated with 100µg/ml of Zymosan, DAMPs supernatant from $0.5X10^6$ CCB fibroblasts, DAMPs debris from $0.5X10^6$ CCB fibroblasts or left untreated. Oxygen radical response was measured by RT-luminol assay during 210 minutes. The graph shows the mean value of the oxygen radical response kinetics (n=6, except for Zymosan where n=5).

**Table 1. Statistical significance of the carp HKL oxygen radical response kinetics measured with RT-luminol assay after stimulation with β-glucans and DAMPs.** Table shows the significant differences between treatments at the different time point measurements, after two-way ANOVA analysis and Bonferroni post-tests. * = $P< 0.05$, ** = $P< 0.01$, *** = $P< 0.001$, ns= no significant difference. Unstim= Unstimulated cells, DAMP.d= DAMPs debris from $0.5X10^6$ CCB fibroblasts, DAMP.s= DAMPs supernatant from $0.5X10^6$ CCB fibroblasts and Zym= Zymosan (100µg/ml).

| | **Minutes post-stimulation** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatments** | **0-6** | **9** | **12-54** | **57** | **60** | **63-66** | **69** | **72** | **75-81** | **84-93** | **96-117** | **120-144** | **147-207** | **210** |
| **Unstim-DAMP.d** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **Unstim-DAMP.s** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | * | ** | * |
| **Unstim-Zym** | ns | ns | *** | *** | *** | *** | *** | *** | ** | * | ns | ns | ns | ns |
| **DAMP.d - Zym** | ns | ns | *** | *** | *** | ** | * | ns | ns | ns | ns | ns | ns | ns |
| **DAMP.s - Zym** | ns | * | *** | ** | * | ns | ns | ns | ns | ns | ns | ns | ns | ns |

### *3.2. Carp head-kidney leukocytes (HKL) produce different oxygen radical species after β-glucan and DAMPs stimulation.*

The ROS production of carp HKL was measured by RT-luminol assay after stimulation with Zymosan and DAMPs supernatant. Oxygen radical scavengers such as SOD and catalase were used to study the type of radicals produced. The results are expressed as the integral of the kinetic curve for each treatment up to 210 minutes (Max RLU).

An increase in the oxygen radicals produced by HKL was observed after stimulation with Zymosan. Treatment of the Zymosan-stimulated HKL with SOD induced a decrease of the oxygen radical production. In contrast, catalase did not inhibit the oxygen radical production by Zymosan-stimulated HKLs (see figure 2).

Stimulation of HKL with DAMP supernatants induced the production of oxygen radicals. When DAMP-stimulated HKL were treated with SOD or catalase, both were found to markedly reduce the production of oxygen radicals (See figure 2).

In conclusion, stimulation of HKL with Zymosan led to the production of superoxide anion mainly. While after stimulation of HKL with DAMP supernatants the production of superoxide anions, hydrogen peroxide and related radicals were detected.

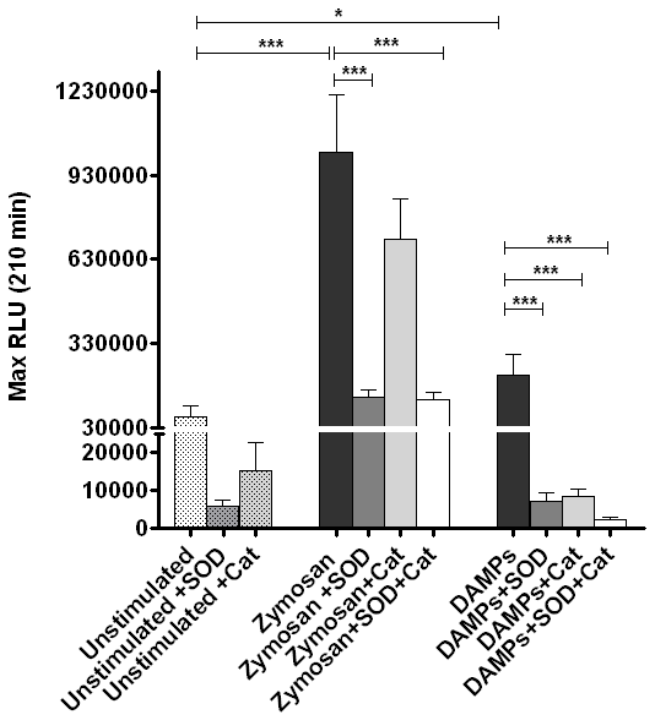


**Figure 2. Carp head-kidney leukocytes (HKL) produce different oxygen radical species after β-glucan and DAMPs stimulation.** Carp HKL were stimulated with 100µg/ml of Zymosan, DAMPs supernatant from $0.5X10^6$ CCB fibroblasts or left untreated. To identify the type of reactive oxygen species produced after stimulation of Zymosan and DAMPs, the oxygen scavengers superoxide dismutase (SOD, 250 U/ml) and catalase (Cat, 300 U/ml) were used. The Oxygen radical response was measured by RT-luminol assay, the graph shows the mean values of the integral of RLU (Max RLU) recorded by the luminometer between 0 and 210 minutes. Error bars represent standard error of the mean, n=8 except for Zymosan+SOD, Zymosan+Cat and Zymosan+SOD+Cat where n=6. Data was statistically analyzed using Mann Whitney test, a table with additional statistical significances is presented as supplementary data. * = $P< 0.05$, *** = $P< 0.001$.

### *3.3. Modulation of wound recovery by CCB fibroblast with β-glucan and hydrogen peroxide during scratch-wound healing in vitro.*

Linear scratches were made in monolayers of CCB fibroblasts and the cultures were stimulated with β-glucans, Hydrogen peroxide, DAMPs supernatant or left untreated. The wound closure was tracked using microscopy pictures as shown in figure 3A. The wound closure data was subsequently retrieved, analyzed with image analysis software and plotted as percentage of wound recovery. Stimulation with low doses of $H_2O_2$ (5 and 10µM) seemed to improve wound recovery during the whole trial period, specially 5µM $H_2O_2$ at day one showed a significant difference when compared to unstimulated fibroblasts. On the other

hand, 300µM of $H_2O_2$ impaired fibroblast growth and cause cell death (see figure 3B). Stimulation of fibroblast cultures with β-glucans and DAMP supernatant did not show a significant effect in wound recovery during the trial period (see figure 3C).

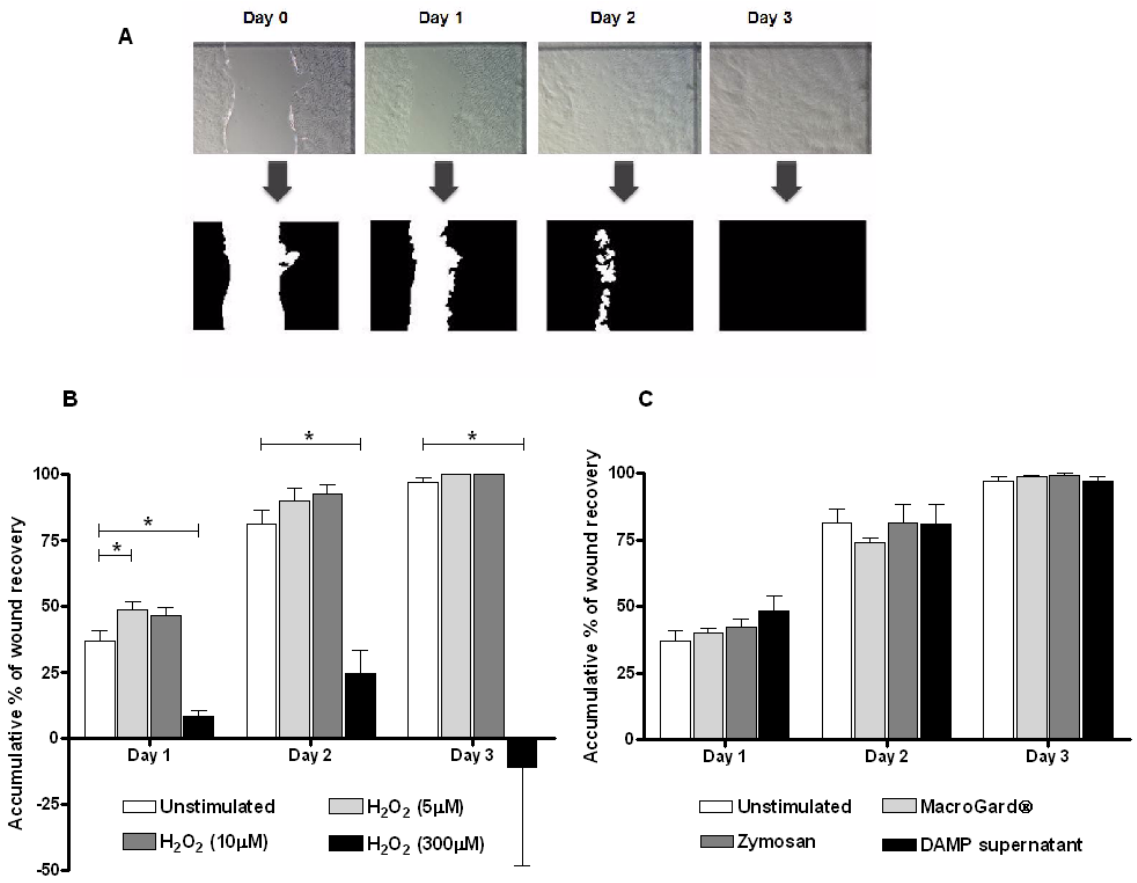


**Figure 3. Modulation of wound recovery by CCB fibroblast with β-glucan and hydrogen peroxide.** Monolayers of CCB fibroblasts were scratches-wounded (≈700 µm) and stimulated with Zymosan (100 µg/ml), 5, 10 or 300 µM Hydrogen peroxide, DAMPs supernatant from 0.5X106 CCB fibroblast or left untreated. The graphs show the mean values of the wound recovery percentage, the error bars represent the standard error of the mean, * = $P< 0.05$. n=6 except for H2O2 (300µM) where n=3. **A)** Follow up of wound closure in untreated scratch-wounded CCB fibroblast cultures. **B)** Accumulative percentage of wound recovery after stimulation with Hydrogen peroxide (5, 10 and 300 µM). **C**) Accumulative percentage of wound recovery after stimulation with MacroGard® (100 µg/ml), Zymosan (100 µg/ml) or the DAMPs supernatant from 0.5X106 CCB fibroblasts. Data was statistically analyzed using Mann Whitney test.

## 4. Discussion

Wound closure and tissue remodelling is very important to keep the health integrity of any organism (Singer and Clark, 1999, Martin, 1997, Noga, 2000). However, the mechanisms by which the organisms can alert and guide the immune system to react appropriately in different scenarios such as during infection or wound healing are not completely understood. In this study the role of oxygen radical species was analysed due to their involvement in

infection clearance and tissue repair processes (Bellavite, 1988, Clifford and Repine, 1982, Rieger and Barreda, 2011). For this purpose, the infection and wound healing scenarios were simulated through the use of the known PAMP Zymosan (Taylor, Tsoni et al., 2007), and fish fibroblast lysates (as representative of DAMPs). These experiments showed that the carp HKL responded with a different pattern of oxygen radical production dependent on the stimulus perceived. Thus, stimulation with Zymosan produced an immediate and strong response, dominated by superoxide anion, consistent with an infection scenario such as the one described for pathogens like *Vibrio anguillarum* (Stave, Roberson et al., 1985), *Aeromonas hydrophila* (Chen and Dexiang, 1991), *Yersinia* ruckeri (Stave, Cook et al., 1987), *Edwardsiella ictaluri* (Waterstrat, Ainsworth et al., 1991). In such cases the main objective is to eradicate the pathogen before it reproduces and affects the host in a greater manner. On the other hand, stimulation with DAMPs elicits a more subtle and long lasting response, dominated by hydrogen peroxide. Mammalian models have suggested the role of oxygen radicals as cellular messengers and stimuli for cellular proliferation when administered in low and continues doses, especially for hydrogen peroxide (Burdon, 1995, Mander, Jekabsone et al., 2006, Burdon, Gill et al., 1996). Likewise, perception of sterile wounds in zebrafish seem to depend on $H_2O_2$ and interestingly, this production of hydrogen peroxide appear to be a result of the Duox oxidase, which is a $H_2O_2$-generating system that does not liberate superoxide (Ameziane-El-Hassani, Morand et al., 2005, Niethammer, Grabher et al., 2009). Our results point in the same direction, showing that in the case of a sterile wound, thus with a lack of an infection threat, the organism would focus in the regeneration of the tissues, rather than in the production of high amounts of oxygen radicals, which are useful to eradicate pathogens, but that can be detrimental to the host causing oxidative stress and tissue damage (Betteridge, 2000, Le Bras, Clément et al., 2005).

Burdon *et al.* showed that addition of 1µM exogenous $H_2O_2$ to baby hamster kidney fibroblast (BHK-21/C13) cultures stimulated cell growth, while a concentration of 100µM was growth inhibitory (Burdon, Alliangana et al., 1995). On the other hand, Mander and co-workers did not found any significant effect on cellular growth after the addition of 1µM exogenous $H_2O_2$ to murine microglia cultures (Mander, Jekabsone et al., 2006). However, culture treatment with ROS generators (such as xhantine and xanthine oxidase) did increase microglia proliferation, even in the presence of a superoxide dismutase scavenger (SOD), indicating therefore the direct involvement of hydrogen peroxide in the cellular proliferation (Mander, Jekabsone et al., 2006). During the current study, addition of single doses of $H_2O_2$

between 0-300 µM did not have any significant effect on fibroblast proliferation (data not shown). However, the slight increase of wound recovery after stimulation of fibroblast cultures with daily low doses of hydrogen peroxide (5µM) emphasizes the importance of this reactive oxygen species, as one of the factors involved in wound healing of common carp. Furthermore, the cellular growth impairment observed after stimulation with 300 µM of $H_2O_2$ shows the importance of ROS quantities and timing during carp wound healing.

Direct β-glucan stimulation of scratched-wounded fibroblast did not enhanced wound recovery percentage. However, previous work of ours showed a faster wound closure in β-glucan stimulated carp (Przybylska, Schmidt et al., 2013). Therefore, β-glucan induced immune modulation of carp wound healing may relay on other cell types (epithelial cells, goblet cells or tissue-residing leukocytes). Stimulation of carp leukocytes with β-glucan produced a pathogen eradication response rather than a tissue regeneration environment. Therefore, the enhanced wound healing response observed after β-glucan stimulation might be ROS-independent or influenced by oxygen scavengers.

In conclusion, β-glucan stimulation of scratch-wounded fibroblasts cultures did not enhance wound recovery. In contrast, low and continuous doses of hydrogen peroxide seemed to improve wound closure. Additionally, it was demonstrated that carp HKL can recognize DAMPS and PAMPs and produce different ROS patterns in response to them. Therefore, ROS patterns might be one of the possible ways in which fish alert the immune system and drive the immune response towards pathogen eradication or tissue repair.

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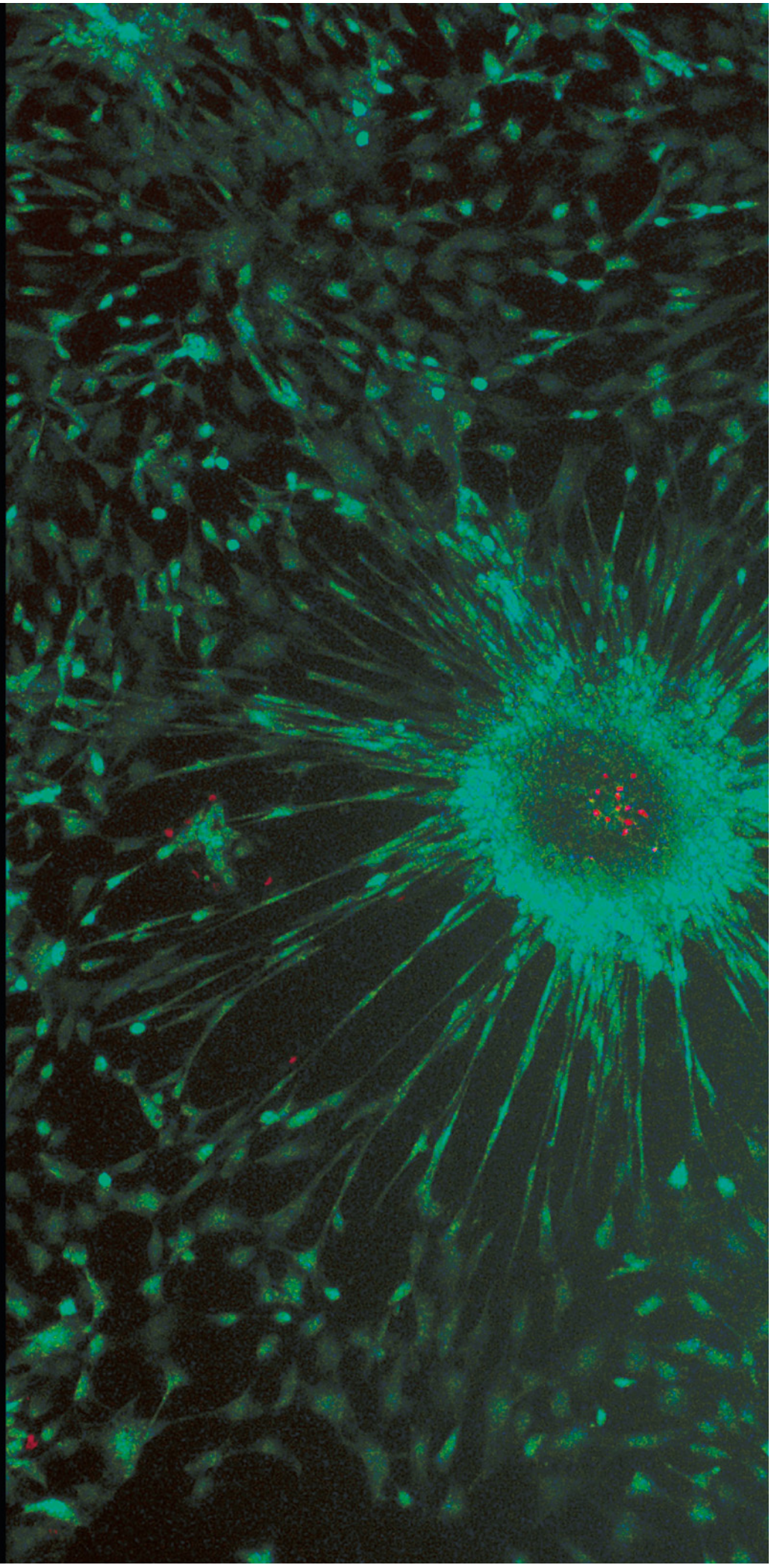
## Suppplementary data

**Table 1. Statistical significance of the carp HKL oxygen radical response**

**different oxygen radical species after β-glucan and DAMPs stimulation.**Table shows the significant differences among treatments after analysis with Mann Withney Test. * = $P< 0.05$, ** = $P< 0.01$, *** = $P< 0.001$, ns= no significant difference. SOD= Superoxide dismutase (250 U/ml), Cat= Catalase (250 U/ml), DAMP supernatnat= DAMPs supernatant from $0.5X10^6$ CCB fibroblasts and Zymosan (100µg/ml).

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **1. Unstimulated** | **1.** | | | | | | | | | |
| **2. SOD** | ** | **2.** | | | | | | | | |
| **3. Cat** | * | ns. | **3.** | | | | | | | |
| **4. Zymozan** | *** | *** | *** | **4.** | | | | | | |
| **5. Zymozan +SOD** | * | *** | ** | *** | **5.** | | | | | |
| **6. Zymozan +Cat** | ** | *** | *** | ns. | ** | **6.** | | | | |
| **7. Zymozan +SOD+Cat** | * | *** | ** | *** | ns. | ** | **7.** | | | |
| **8. DAMP supernatant** | * | *** | ** | ** | ns. | * | ns. | **8.** | | |
| **9. DAMP supernatant +SOD** | ** | ns. | ns. | *** | *** | *** | *** | *** | **9.** | |
| **10. DAMP supernatant +Cat** | ** | ns. | ns. | *** | *** | *** | *** | *** | ns. | **10.** |
| **11. DAMP supernatant +SOD+Cat** | *** | ns. | ** | *** | *** | ** | ** | *** | ns. | * |



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